#37
B. Denny
12/29/95

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Ser. No.:	08/315,673	§	Docket No. D-5050-C3
		§	
Filing Date:	09/30/94	§	Examiner: A. Marschel
		§	
Applicant:	Caskey, et al.	§	Art Unit: 1807
		§	
Title:	Multiplex Genomic DNA Amplification for Deletion Detection	§	

Box Non-Fee Amendment
Assistant Commissioner of Patents
Washington, D.C. 20231

DECLARATION OF JEFFREY CHAMBERLAIN UNDER 37 C.F.R. 1.132

Dear Sir:

I Jeffrey Chamberlain, Ph.D., do hereby depose and say as follows:

1. I am an Associate Professor in the Department of Human Genetics at the University of Michigan Medical School. I am skilled in the field of molecular biology, specifically, I am skilled in the field of multiplex DNA amplification using the polymerase chain reaction. My *curriculum vitae* is attached hereto as Exhibit A.

2. I am a co-inventor of the invention that is the subject of U.S. Serial No. 08/315,673, filed September 30, 1994. I have read U.S. Serial No. 08/315,673 and I am aware of its contents.

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to Assistant Commissioner of Patents Washington, D.C. 20231 on Oct. 3, 1995
Linda A. Bourg
Linda A. Bourg 10/3/95
Signature Date

3. I have been involved personally in the investigation described in the above-referenced patent application. The following experiments were performed at my direction and the following comments are based on my experience with multiplex DNA amplification:

Having a narrow range of Tms for the primers is probably the single most important aspect of the multiplex DNA amplification method of the present invention. Tms are inherent with a primer, and choosing primers with similar Tms is critical to a successful multiplex reaction.

The uniqueness of the sequences with which a primer anneals is critical to obtaining a specific reaction product. One cannot interpret a reaction, when one needs to observe the presence or absence of a product to interpret the results of the reaction, unless the reaction products are specific and reproducible. As stated in the specification on page 16, lines 15-34, "the temperature is dependent on the uniqueness of the primer sequence." Later in the same paragraph it reads "GC rich primers need higher temperatures to prevent cross hybridization and to allow unique amplification." Uniqueness of annealing requires that a primer be near the melting temperature. In the context of a PCR this is even more important than might otherwise be. In PCR, one needs two primers to anneal uniquely for a successful reaction. If one primer anneals uniquely, but the other does not, one might still have a successful PCR. This is because you need two primers to anneal to a DNA sequence, pointed towards each other, and at the proper distance apart to support amplification, in order to have a successful PCR. If one primer binds to multiple places, but the second primer does not, one may still have a successful PCR as the misannealing primer will not support *exponential* amplification, only a linear amplification. Linear amplification does not produce detectable product after 30 cycles (2 x 30 = 60 fold increase in yield, as opposed to 2^{30}). However, as more and more primers are added to the reaction, more primers may misanneal, and the odds increase rapidly that a reaction will no longer work or render interpretable results. This is why we, the inventors of the present

invention, observed that the Tm of the primers must be similar to enable a successful reaction to occur.

4. Koenig *et al* supply a cDNA sequence, which is the sequence derived from an mRNA copy, not a gene sequence. Our primer pairs are not derived from the cDNA sequence, but were derived from intron sequences which cannot be predicted from a cDNA sequence. A cDNA sequence is composed entirely of exon sequences. We cloned portions of the gene in order to discover the introns and determine their sequences. The intron data generated by us was novel and we used it to make PCR primers. Koenig *et al.* do point out the location of a few introns; however, Koenig et al. show less than twenty percent of those in the gene. Additionally, the predicted location of many of the introns shown by Koenig et al. was incorrect; indeed, we demonstrated the errors in a later publication (*see* Baumbach et al., *Neurology* 39:465-74 (1989), attached hereto as Exhibit B). Koenig erroneously reported the location of many of the introns that we eventually cloned and sequenced. Thus, Koenig did not supply us with the needed sequence data, nor even the proper location in the gene to search for the sequence data we needed.

5. As the person signing this declaration, I hereby declare that all statements herein of my own knowledge are true, that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the applications or any patent issued therein.

Date: Sept. 27, 1995

Dr. Jeffrey S. Chamberlain, inventor

Curriculum Vitae

PERSONAL DATA

Name: Jeffrey S. Chamberlain
Social Security Number: 526-17-7817

EDUCATION

1971-1974	Clear Creek High School, League City, Texas	
1974-1978	Rice University, Houston, Texas	B.A., Biochemistry
1978-1985	University of Washington, Seattle, Washington Graduate advisor: Dr. Stephen D. Hauschka.	Ph.D., Biochemistry
1985-1990	Postdoctoral Fellow with Dr. C.T. Caskey, Institute for Molecular Genetics Baylor College of Medicine, Houston, Texas	

ACADEMIC APPOINTMENTS

1990-1994	Assistant Professor, Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Michigan
1994-	Associate Professor, Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Michigan, 48109-0618.

SCIENTIFIC ACTIVITIES

Editorial Boards

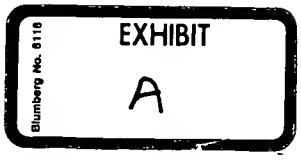
1990-	*PCR Methods and Applications* (Cold Spring Harbor Laboratory Press)
1993-	*Gene Therapy* (MacMillan Publishing Company)
1995-	*Genome Research* (Cold Spring Harbor Laboratory Press).
1995-	*Human Molecular Genetics* (Oxford University Press).

Study Sections

1989-1995	Muscular Dystrophy Association, Fellowship Review Subcommittee
1991	NIH, Site Visit Team, NIAMS Program Project Grant, U. Washington
1992	NIH, NHLBI Ad Hoc Review Committee for the Genetics of Asthma
1993-	NIH, Policy and Monitoring Board, Genetics of Asthma Multicenter Study Group.
1993-	Muscular Dystrophy Association, Task Force on Therapy for Genetic Diseases
1995	NIH NIAMS, Ad Hoc Reviewer, U. Penn Program Project Grant
1995-	NIH, Molecular Cytology Study Section, Ad Hoc Reviewer
1995-	Muscular Dystrophy Association, Scientific Advisory Committee
1995-	National Science Foundation, Advisory Committee on the Human Genome Diversity Project.
1995	NIH NIAMS, Ad Hoc Reviewer for R29 Grant Applications

HONORS AND AWARDS

1986-1988	Muscular Dystrophy Association Postdoctoral Fellowship
1991-1994	Basil O'Conner Starter Scholar Research Award, March of Dimes Birth Defects Foundation.
1994	Research Partnership Award, University of Michigan.
1994	Service Merchandise Inc., Leadership Award For Neuromuscular Disease Research
1995	Gift of Hope Award, Jones Intercable Inc. and The New Mexico Muscular Dystrophy Association.
1995	Faculty Recognition Award, University of Michigan.



MEMBERSHIPS AND OFFICES IN PROFESSIONAL SOCIETIES

American Association for the Advancement of Science
American Society of Human Genetics
American Society of Microbiology

TEACHING ACTIVITIES

1990-1991	Human Genetics 803, taught one-third of the class
1992-	Human Genetics 541, teach one-fourth of the class
1993-	Human Genetics 542, lecture on Duchenne muscular dystrophy
1994-	Human Genetics 804, teach one-third of class

Students and doctoral committees

Post-doctoral Fellows: Current: Dr. Michael Hauser, Dr. Andrea Amalfitano, Dr. Rajendra Kumar-Singh; Former: Dr. Kathy Corrado, 10/91-9/93 presently a Research Fellow with Dr. Kuan Wang, at U.Texas, Austin; **Graduate Students:** Jill Rafael, Carey Lumeng (MSTP), Shian Huey Chiang; Former: Dr. Gregory Cox, 9/90-9/94, currently a post-doctoral Fellow at The Jackson Labs with Dr. Wayne Frankel, Bar Harbor, ME. Dr. Andrea Maichele, 9/90-2/95, currently a post-doctoral Fellow with Manfred Killimann, Ruhr University, Bochum, Germany; Jennifer Wang (Summer Medical Student). **Thesis committees:** Human Genetics students, (current) Stephanie Nelson, George Pappas, Rodney Wiltshire, Rebecca Tucker, Dawn Watkins-Chow, Wonhee Jang, Nicholas Plummer, Doug Mortlock, Ataç Turkay; (past) Scott Keller, Lisa Smit, Eric Burright, German Pasteris, Elizabeth Allen, Sheryl Jankowski, Marion Buckwalter. Students from other departments: Larry D. Adams (Biochemistry), Kam Hunter (Physiology); (past) Everett Chen, Phil Cacheris, Trent Clark (CMB), Kenneth Chahine (Biochemistry) and Martin Albert (Mathematics). **Undergraduates:** Tressa Hutchinson; Former: Ericka Adkins, Victoria Brown, Patti Mills, Chou Truong, William Halliday, Dianne Rumpel, Virginia Willour, Eecole Copin,.

Interdisciplinary Programs: Cell and Molecular Biology Program; Medical Scientist Training Program; Tissue Engineering Program; Presidential Initiative on Organogenesis.

EXTRAMURAL INVITED PRESENTATIONS

"Expression of the murine Duchenne muscular dystrophy gene in the muscle and brain of normal and mutant *mdx* mice," Invited speaker at the UCLA Symposia on Cellular and Molecular Biology of Muscle Development, April 3-10, 1988, Steamboat Springs, CO.

"Multiplex amplification for diagnosis of Duchenne muscular dystrophy," Invited speaker at The Banbury Center Conference on The Polymerase Chain Reaction, December 11-14, 1988, The Banbury Center, New York.

"Tissue specific expression of the murine dystrophin gene," Invited speaker at The Banbury Center Conference on Dystrophin, April 2-5, 1989, The Banbury Center, New York.

"Strategies for Mutation Detection in Duchenne Muscular Dystrophy," Department of Genetics, Hospital for Sick Children, University of Toronto, Toronto, Ontario, April 26, 1989.

"Characterization of the Central Deletion Prone Region of the Duchenne Muscular Dystrophy Locus," Ontario Cancer Institute, Toronto, Ontario, April 25, 1989.

"Molecular Genetics Duchenne Muscular Dystrophy," Department of Neurology, Ohio State University, Columbus, OH, July 14, 1989.

"Molecular Genetics Duchenne Muscular Dystrophy," Pasteur Institute, Paris, France, October 2, 1989.

"Molecular Genetics Duchenne Muscular Dystrophy," G. Gaslini Institute, Genoa, Italy, October 4, 1989.

"Molecular Genetics Duchenne Muscular Dystrophy," Department of Genetics, Sylvius Laboratories, Leiden, Netherlands, October 6, 1989.

"Expression and mutation of the Duchenne muscular dystrophy gene," Invited Lecture at The American Association of Clinical Chemistry meeting, The Fourth San Diego Conference on "Nucleic Acid Applications", October 25-28, 1989; San Diego, CA

"PCR Methods for the Analysis of Dystrophin Expression in *mdx* Mice," Invited speaker at The UCLA Symposium on Tissue Engineering, April 6-12 1990, Keystone, CO.

"Automated Approaches for the Diagnosis of Duchenne Muscular Dystrophy," Applied Biosystems Inc., Foster City, California, July 23, 1990.

"Characterization of the Gene for the Gamma Subunit of Mouse Phosphorylase Kinase," Institute for Physiological Chemistry, Rühr University, Bochum, Germany, September 17, 1990.

"Approaches for Diagnosis and Gene Therapy of Duchenne Muscular Dystrophy," Howard Hughes Medical Institute, University of Iowa, Iowa City, Iowa, October 8, 1990.

"Approaches for Diagnosis and Gene Therapy of Duchenne Muscular Dystrophy," Lecturer at symposium on 'Neuromuscular Development, Function and Dysfunction', Department of Neurology, McGill University, Montreal, Canada, October 22, 1990.

"Expression of the Duchenne Muscular Dystrophy Gene in Muscle and Non-Muscle Tissues," Department of Biochemistry, University of Washington, Seattle, Washington, November 15, 1990.

"Expression of the Duchenne Muscular Dystrophy Gene in Muscle and Non-Muscle Tissues," Department of Genetics, Hospital for Sick Children, University of Toronto, Toronto, Ontario, June 19, 1991.

"Expression of dystrophin isoforms in the central nervous system," Invited speaker at The 23rd Annual March of Dimes Clinical Genetics Conference, July 7-10, 1991, Vancouver, British Columbia, Canada.

Approaches for Gene Therapy of Duchenne Muscular Dystrophy," Department of Genetics, Wayne State University, Detroit, MI, December 5, 1991.

"Mapping the Gene for Familial Breast Cancer," Chromosome 17 Workshop, Park City Utah, March 13, 1992.

"Multiplex PCR," Keynote Speaker, Perkin Elmer Symposium: "The Polymerase Chain Reaction", Chicago, IL, April 27, 1992.

"Approaches for gene therapy of Duchenne muscular dystrophy," Invited speaker at The EMBO workshop on *Molecular biology and pathology of skeletal and cardiac myogenesis*, Sept 26-Oct 1, 1992, Alghero, Italy.

"Correction of Duchenne Muscular Dystrophy in Transgenic *Mdx* Mice," Department of Chemistry, Northern Illinois University, Dekalb, IL, November 20, 1992.

"Elimination of Dystrophic Symptoms in Transgenic *Mdx* Mice," Department of Anatomy, University of Wisconsin, Madison, WI, March 23, 1993.

"Prospects for Gene Therapy of Duchenne Muscular Dystrophy," Muscular Dystrophy Association Gene Therapy Workshop, New York City, April 22, 1993.

"Expression of the Dystrophin Gene in Normal, *Mdx*, and Transgenic Mice," The Banbury Conference on "Gene Reactivation as a Therapeutic Strategy for Duchenne Muscular Dystrophy, The Banbury Center, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, April 25, 1993.

"The Impact of the Human Genome Project on the Treatment of Genetic Diseases," International Symposium on the Human Genome, Mexico City, Mexico, May 8, 1993.

"Molecular Genetics of Duchenne Muscular Dystrophy", International Symposium on the Human Genome, Oaxtepec, Mexico, May 9, 1993.

"Functional Studies of Dystrophin Isoforms in Transgenic *mdx* Mice" Institute for Molecular Genetics, Baylor College of Medicine, Houston, Texas, December 23, 1993.

"Development of dystrophin expression vectors for gene therapy of Duchenne muscular dystrophy", Muscular Dystrophy Association Symposium on Therapy for Genetic Diseases, January 24-25, Tucson, Arizona.

"Ethical Considerations in the Development of Gene Therapy for Muscular Dystrophy", Dilemmas and Resolutions: Research with Children as Subjects. Conference sponsored by the University of Texas-Houston Health Science Center, February 28 -March 1, 1994.

"Transgenic Models for Gene Therapy of DMD", Department of Biochemistry, University of Texas, March 2, 1994.

"Analysis of dystrophin expression vectors in *mdx* mice: implications for gene therapy of DMD". Cambridge Healthtech Institute Meeting on Gene Therapy, Washington D.C., April 25, 1994.

"Approaches for Gene Therapy of Duchenne Muscular Dystrophy", North Ann Arbor Rotary Club, May 5, 1994.

"DNA Diagnostics with di- and tetra-nucleotide repeat polymorphisms: Duchenne muscular dystrophy and Breast cancer". Cambridge Healthtech Institute meeting on 'The Polymerase Chain Reaction', San Francisco, June 8-10, 1994.

"Approaches for Gene Therapy of Duchenne Muscular Dystrophy", Department of Neurology, Wayne State University Medical School, Detroit, MI, August 19, 1994.

"Development of dystrophin expression vectors for gene therapy of muscular dystrophy", The UpJohn Company, Kalamazoo, Michigan, August 26, 1994.

"Dystrophin expression vectors for gene therapy of muscular dystrophy", Michigan State University, Symposium on Advances in Gene Therapy, Lansing, Michigan, September 10, 1994.

"An in-depth look at PCR ", Session Chair for symposium on 'A Decade of PCR - In Celebration of Ten Years of Amplification", Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, September 12-14, 1994.

"The applicability of gene transfer therapy for Duchenne muscular dystrophy", The Child Neurology Society and International Child Neurology Association Conjoint Meeting, San Francisco, California, October 2-8, 1994.

"Development of truncated dystrophin clones for gene therapy of DMD", IBC Conference on Gene Therapy, November 14-15, 1994, Washington, DC.

"Development and testing of dystrophin expression vectors", Muscular Dystrophy Association Workshop on Gene Therapy for Duchenne Muscular Dystrophy, Tucson, Arizona, November 18-19, 1994.

"Molecular genetics of Duchenne muscular dystrophy", Depts. of Neurology and Pediatrics Grand Rounds, University of New Mexico, Albuquerque, New Mexico, January 19 and 20, 1995.

"Mutational analysis of dystrophin cDNAs", Institute for Human Genetics, University of Minnesota, Minneapolis, February 23, 1995.

"Prospects for gene therapy of Duchenne muscular dystrophy", Cold Spring Harbor Laboratory Meeting on Duchenne muscular dystrophy, Cold Spring Harbor, NY, February 26, 1995.

"Functional studies of dystrophin domains in transgenic *mdx* mice" Dept. of Physiology, University of North Carolina, Chapel Hill, March 13, 1995.

"Analysis of dystrophin function in transgenic *mdx* mice" Symposium on Muscular Dystrophy, Texas Tech University Health Sciences Center, Lubboch, April 6, 1995.

"Gene mapping and molecular diagnostics" 2nd International Conference on Pharmaceutical Biotechnology, Gent, Belgium, April 23-27, 1995.

"Muscular dystrophy in transgenic mdx mice: functional analysis of dystrophin" Annual Meeting of The American Association of Neuropathologists; Special Course: Transgenic animals to model human diseases of the nervous system, San Antonio, TX, June 7, 1995.

"Development of Dystrophin Expression Vectors for Gene Therapy of Muscular Dystrophy" Mayo Clinic, Rochester, MN, September 1, 1995.

"Update on Research into Muscular Dystrophy" Jerry Lewis Labor Day Telethon for the Muscular Dystrophy Association, Los Angeles, CA., Sept 4, 1995.

"Understanding the Function of Dystrophin: Application to Gene Therapy of Muscular Dystrophy" Department of Cellular & Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, TX, September 21, 1995.

COMMITTEE AND ADMINISTRATIVE SERVICE

University of Michigan

1994	Pew Higher Education Roundtable
1995-	Provost's Faculty Oversight Committee on Value Centered Management

Medical School

1990-1993	University of Michigan Gene Therapy Program Project, Executive Committee member, co-investigator
1990-	University of Michigan Human Genome Center, Executive Committee member, co-investigator, and Director of Genetic Marker and (1994-) DNA Sequencing Core Laboratories
1992-	Diabetes Research and Training Center, Molecular Biology Core Advisory Committee
1993-	Multipurpose Arthritis and Musculoskeletal Disease Center, Transgenic Mouse Core Lab Oversight Committee
1993	Dean's Faculty Focus Group on Future Directions of the Medical School
1994-	Dean's Task Force on Organogenesis
1994-	Biomedical Research Council (BMRC) Member
1995-	Symposium and Seminar Committees, Presidential Initiative on Organogenesis

Department of Human Genetics

1991-	Special Events Committee
1994-1995	Self Study Committee for The Department of Human Genetics
1994-	Awards Committee Chair
1995-	Chair's Advisory Committee
1995-	Faculty Search Committee

BIBLIOGRAPHY

Completed Publications in Scientific Journals
Peer-Reviewed Publications

1. Schroepfer GJ Jr, Monger D, Taylor AS, Chamberlain JS, Parish EJ, Kisic A and Kandutsch AA: Inhibitions of sterol synthesis. Hypocholesterolemic action of 5-α-cholest-8(14)-ene-3β-ol-15-one in rats and mice. Biochem Biophys Res Commun 1977; 78:1227-1233.
2. Hauschka SD, Lim R, Clegg CH, Chamberlain JS and Linkhardt T: Cell replication, mitogen receptors, and regulation of commitment to terminal differentiation by mitogens. Cell Biol Internat Rep 1983; 7:553-554.
3. Hauschka SD, Lim R, Clegg C, Chamberlain J, Bulinski J and Linkhardt T: Trophic influences on developing muscle. Adv Exp Med Biol 1985; 182:113-122.
4. Chamberlain JS, Jaynes JB and Hauschka SD: Regulation of creatine kinase induction in differentiating mouse myoblasts. Mol Cell Biol 1985; 5:484-492.
5. Jaynes, JB, Chamberlain JS, Buskin J and Hauschka SD: Transcriptional regulation of the muscle creatine kinase gene and regulated expression in transfected mouse myoblasts. Mol Cell Biol 1986; 6:2855-2864.
6. Buskin JN, Jaynes JB, Chamberlain JS and Hauschka SD: The mouse muscle creatine kinase cDNA and deduced amino acid sequences: comparison to evolutionary related enzymes. J Mol Evol 1986; 22:334-341.
7. Chamberlain JS, VanTuinen P, Reeves AA, Philip BA and Caskey CT: Isolation of cDNA clones for the catalytic γ-subunit of mouse muscle phosphorylase kinase: Expression of mRNA in normal and mutant Phk mice. Proc Natl Acad Sci USA 1987; 84:2886-2890.
8. Chamberlain JS, Grant S, Reeves AA, Mullins L, Stephenson D, Hoffman EP, Monaco AP, Kunkel LM, Caskey CT and Chapman VM: Regional localization of the murine Duchenne muscular dystrophy locus on the mouse X chromosome. Somat Cell Molec Genet 1987; 13:671-678.

9. Chamberlain JS, Pearlman JA, Muzny DM, Gibbs RA, Ranier JE, Reeves AA and Caskey CT: Expression of the murine Duchenne muscular dystrophy gene in muscle and brain. Science 1988; 239:1416-1418.
10. Hoffman EP, Fischback K, Brown RH, Johnson LM. Medori R, Loike JD, Harris JB, Waterston R, Brook M, Specht L, Kupsky R, Chamberlain JS, Caskey CT, Shapiro F and Kunkel LM: Characterization of dystrophin in muscle-biopsy specimens from patients with Duchenne's or Becker's muscular dystrophy. N Engl J Med 1988; 318:1363-1368.
11. Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN and Caskey CT: Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res 1988; 16:11141-11156.
12. McCabe ERB, Towbin J, Chamberlain J, Baumbach L, Witkowski J, van Ommen GJB, Koenig M, Kunkel LM and Seltzer WK: cDNA probes for the Duchenne muscular dystrophy locus demonstrate a previously undetectable deletion in a patient with dystrophic myopathy, glycerol kinase deficiency, and congenital adrenal hypoplasia. J Clin Inv 1989; 83:95-99.
13. Baumbach LL, Chamberlain JS, Ward PA, Farwell NJ and Caskey CT: Molecular and clinical correlations of deletions leading to Duchenne and Becker muscular dystrophy. Neurology 1989; 39:465-474.
14. Towbin JA, Wu D, Chamberlain JS, Larsen PD, Seltzer WK and McCabe ERB: Characterization of patients with glycerol kinase deficiency (GKD) utilizing cDNA probes for the Duchenne muscular dystrophy (DMD) locus. Hum Genet 1989; 83:122-126.
15. Gillard EF, Chamberlain JS, Murphy EG, Duff CL, Smith B, Burghess AHM, Thompson MW, Sutherland J, Oss I, Bodrug S, Klamut HJ, Ray PN and Worton RG: Molecular and phenotypic analysis of patients with deletions within the deletion-rich region of the DMD gene. Am J Hum Genet 1989; 45:507-520.
16. Grompe M, Gibbs RA, Chamberlain JS and Caskey CT: Detection of new mutation diseases in man and mouse. Mol Biol Med 1990; 6:511-521.
17. Ballabio A, Ranier JE, Chamberlain JS and Caskey CT: Screening for steroid sulfatase gene deletions *via* multiplex DNA amplification. Hum Genet 1990; 84:571-573.
18. Towbin JA, Chamberlain JS, Wu D, Pillers D, Seltzer WK and McCabe ERB: DXS 28 (C7) maps centromeric to DXS 68 (L1-4) and DXS 67 (B24) by deletion analysis. Genomics 1990; 7:442-444.
19. Pillers DM, Towbin JA, Chamberlain JS, Wu D, Ranier JE, Powell BR and McCabe ERB: Deletion mapping of Aland Island eye disease to Xp21 between DXS67 (B24) and Duchenne muscular dystrophy. Am J Hum Genet 1990; 47:795-801.
20. Lee CC, Pearlman JA, Chamberlain JS and Caskey CT: Expression of recombinant dystrophin and its localization to the cell membrane. Nature 1991; 349:334-336.
21. Chamberlain JS, Farwell NJ, Ranier JE, Cox GA and Caskey CT: PCR analysis of dystrophin gene mutation and expression. J Cell Bioc 1991; 46:255-259.
22. Blonden LAJ, Grootscholten PM, den Dunnen JT, *et al.* (39 authors, includes Chamberlain JS): Breakpoints in the 200-kb deletion-prone P20 region of the DMD gene are widely spread. Genomics 1991; 10:631-639.
23. Mayrand PE, Robertson J, Ziegle J, Hoff LB, McBride LJ, Chamberlain JS and Kronick MN: Automated genetic analysis. Annales de Biologie Clinique 1991; 4:224-230.
24. Hoffman EP, Garcia CA, Chamberlain JS, Angelini C, Lupski JR and Fenwick R: Is the Carboxyl-terminus of dystrophin required for membrane association? A novel, severe case of Duchenne muscular dystrophy. Annals Neurol 1991; 30:605-610.
25. Clemens PR, Fenwick RG, Chamberlain JS, Gibbs RA, de Andrade M, Chakraborty R and Caskey CT: Carrier detection and prenatal diagnosis in Duchenne and Becker muscular dystrophy families, using dinucleotide repeat polymorphisms. Am J Hum Genet 1991; 49:951-960.
26. Bies RD, Phelps S, Cortez MD, Roberts R, Caskey CT and Chamberlain JS: Human and murine dystrophin mRNA transcripts are differentially expressed during skeletal muscle, heart, and brain development. Nucleic Acids Res 1992; 20:1725-1731.

27. Chamberlain JS, Chamberlain JR, Fenwick RG, *et al.*: Diagnosis of Duchenne and Becker muscular dystrophies by polymerase chain reaction: A multicenter study. JAMA 1992; 267:2609-2615.
28. Maichele AJ and Chamberlain JS: Cross species conservation of a polymorphic dinucleotide repeat in the 3' untranslated region of the dystrophin gene. Mamm Genome 1992; 3:290-292.
29. Worley KC, Towbin JA, Zhu XM, Barker DF, Ballabio A, Chamberlain JS, Biesecker LG, Blethen SL, Brosnan P, Fox JE, Rizzo WB, Romeo G, Sakuragawa N, Seltzer WK, Yamaguchi S and McCabe ERB: Identification of new markers in Xp21 between DXS28 (C7) and DMD. Genomics 1992; 13:957-961.
30. Houzelstein D, Lyons GE, Chamberlain JS and Buckingham ME: Localization of dystrophin gene transcripts during mouse embryogenesis. J Cell Biol 1992; 119:811-822.
31. Chamberlain JS, Boehnke M, Frank TS, Kiousis S, Xu J, Guo S-W, Hauser ER, Norum RA, Helmbold EA, Markel DS, Keshavarzi SM, Jackson CE, Calzone K, Garber J, Collins FS and Weber BL: BRCA1 maps proximal to D17S579 on chromosome 17q21 by genetic analysis. Am J Hum Genet 1993; 52:792-798.
32. Maichele AJ, Farwell NJ and Chamberlain JS: A B2 repeat insertion generates alternate structures of the mouse muscle γ-Phosphorylase kinase gene. Genomics 1993; 16:139-149.
33. Cox GA, Phelps SF, Chapman VM and Chamberlain JS: New *mdx* mutation disrupts expression of muscle and nonmuscle isoforms of dystrophin. Nature Genet 1993; 4:87-93.
34. Towbin JA, Hejtmancik JF, Brink P, Gelb B, Zhu XM, Chamberlain JS, McCabe ERB and Swift M: X-linked dilated cardiomyopathy (XLCM): Molecular genetic evidence of linkage to the Duchenne muscular dystrophy (dystrophin) gene at the Xp21 locus. Circulation 1993; 87:1854-1865.
35. Ting C-N, Burgess DL, Chamberlain JS, Keith TP, Falls K and Meisler MH: Phosphoenolpyruvate carboxykinase (GTP): Characterization of the human PCK1 gene and location distal to MODY on chromosome 20. Genomics 1993; 16:698-706.
36. Cox GA, Cole NM, Matsamura K, Phelps SF, Hauschka SD, Campbell KP, Faulkner JA and Chamberlain JS: Overexpression of dystrophin in transgenic *mdx* mice eliminates dystrophic symptoms without toxicity. Nature 1993; 364:725-729.
37. Maichele AJ and Chamberlain JS: The γ-Phosphorylase kinase gene, *Phkg*, maps to murine chromosome 5 near *Gus*. Mamm Genome 1994; 5:15-18.
38. Corrado K, Mills P, Chamberlain J: Deletion analysis of the dystrophin actin-binding domain. FEBS Letts 1994; 344:255-260.
39. Denetclaw WF, Hopf WF, Cox GA, Chamberlain JS, and Steinhardt RA: Transgenic *mdx* mice expressing full-length dystrophin show normal calcium regulation. Molec Biol Cell 1994; 5:1159-1167.
40. Rafael JA, Sunada S, Cole NM, Campbell KP, Faulkner JF, and Chamberlain JS: Prevention of dystrophic pathology in *mdx* mice by a truncated dystrophin isoform. Hum Molec Genet 1994; 3:1725-1733.
41. Couch FJ, Kiousis S, Castilla LH, Xu J, Chandrasekharappa SC, Chamberlain JS, Collins FS, Weber BL: 1994. Characterization of ten new polymorphic dinucleotide repeats and generation of a high-density microsatellite based physical map of the BRCA1 region of chromosome 17q21. Genomics 1994; 24:419-424.
42. Cox GA, Sunada Y, Campbell KP, Chamberlain JS. Dp71 can form a stable dystrophin-associated glycoprotein complex in muscle but fails to prevent dystrophy. Nature Genet 1994; 8:333-338.
43. Flejter W, Bennett-Baker P, Barcroft C, Kiousis S, and Chamberlain J: Identification of cosmids containing STRPs specific to 17q21 using chromosome microdissection and FISH. Genomics 1995; 25:413-420.
44. Yang B, Jung D, Rafael JA, Chamberlain JS, and Campbell KP: Identification of α-syntrophin binding to syntrophin triplet, dystrophin, and utrophin. J Biol Chem 1995; 270:4975-4978.
45. Merajver SD, Frank TS, Xu J, Pham TM, Calzone KA, Bennett-Baker P, Chamberlain J, Boyd J, Garber JE, Collins FS, Weber BL: Germline BRCA1 mutations and loss of the

wild-type allele in tumors from families with early onset breast and ovarian cancer. Clin Cancer Res 1995;1:539-544.
46. Passos-Bueno MR, Moreira ES, Vainzof M, Chamberlain J, Marie SK, Pereira L, Akiyama J, Roberds SL, Campbell KP, Zatz M: A common missense mutation in the adhalin gene in three unrelated Brazilian families with a relatively mild form of autosomal recessive limb-girdle muscular dystrophy. Hum.Mol.Genet.;4:1163-1167.
47. Phelps SF, Hauser MA, Cole NM, Rafael JA, Hinkle RT, Faulkner JA, Chamberlain JS: Expression of full-length and truncated dystrophin mini-genes in transgenic *mdx* mice. Hum.Mol.Genet;4:1251-1258.

Non Peer-Reviewed
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Molecular and clinical correlations of deletions leading to Duchenne and Becker muscular dystrophies

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Article abstract—Human DMD cDNA probes have been used to delineate possible deletions in 160 affected males. Approximately 56% of these individuals had detectable deletions, 29% of which mapped to a region centered around 500 kb from the 5′ end of the gene whereas 69% mapped to a region located centrally 1,200 kb from the 5′ end. We have observed no correlation between the extent of a deletion, its location, and clinical severity of the associated disease. For some cases with deletions in the two high-frequency deletion regions, the predicted effect upon translational reading frame of the resultant dystrophin mRNA did not correlate with the associated disease phenotype.

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Duchenne muscular dystrophy (DMD) is a severe X-linked disease with an incidence of 1 in 3,500 males.[1] Approximately ⅓ of these cases result from a new mutation[2]. DMD is allelic with Becker muscular dystrophy (BMD), a clinically similar but less severe form of dystrophy affecting 1 in 30,000 males.[1,3]

Until recently, the most accurate diagnostic tests for DMD were the determination of serum creatine kinase

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levels, muscle biopsy, and EMG.[4] However, the application of recombinant DNA technology to the diagnosis of DMD has resulted in the development of more accurate tests. With the use of recently isolated human cDNAs for the DMD gene,[5-7] deletions throughout the DMD locus are now detected in over 50% of all cases.[5-8] We report here our experience with deletion detection in 160 affected males using the human DMD cDNAs and present a detailed molecular characterization of deletions that encompass regions of known gene structure. We have observed no correlation between the deletion size or location and the clinical severity of the disease. In the course of this study, we have also determined the exon/intron structure of the deletion-prone central region of the DMD gene. This data together with published DNA sequence information of 5′ exons permits predictions concerning the effect of deletions upon the translation of dystrophin mRNA in these patients. Analysis of 36 patients with known clinical phenotypes reveals paradoxes between these phenotypes and the predicted translational reading frames of dystrophin mRNA.

Patients and methods. Blood was obtained from 160 affected males referred to the Robert J. Kleberg DNA Diagnostic Laboratory as part of familial prenatal diagnostic cases. A differential diagnosis of DMD or BMD was made based on the clinical progression of the muscular dystrophy as indicated in patient medical records. We have applied established criteria for DMD diagnosis,[1,9] and have expanded the classification scheme to include patients with an intermediate phenotype between DMD and BMD. A patient was categorized as having DMD if he was diagnosed between the ages of 1 and 7 years, wheelchair-dependent by age 15, and either alive through his mid-20s or deceased due to complications of the illness. A patient was classified as having BMD if he was diagnosed later than age 7, wheelchair-dependent past 15 years of age, and still living without life support systems in his late 20s to mid-30s. If the disease progression of a patient was in between the above 2 criteria, then he was classified as an intermediate (BMD/DMD) disease phenotype. This category corresponds to mild DMD or severe BMD patients, also known as "outliers."[10]

Analysis of affected individuals using cDNA probes was conducted after isolating DNA from the white blood cells of 8 to 15 ml of heparinized blood as described.[11,12] cDNA probes were labeled to a specific activity of greater than 1×10^9 cpm/μg DNA using random oligonucleotide primers[13] and Southern analysis of *Hin*dIII digested genomic DNA was performed with the radiolabeled probes at 2×10^6 cpm/ml. The cDNAs used in these studies were the 9-7, 30-2, 30-1, and 44-1 clones, initially reported as clones 1a+1b+2, 2+3, 4+5a, and 8, respectively.[5] Two probes were subcloned from the 2.6 kb fragment spanning the approximate region of nucleotides 4,500-6,100 (originally reported as clones 5b+6). This region was divided into 2 fragments by *Hin*cII digestion, the 5′ 2.0 kb-fragment termed 47-4A and the 3′ 0.6 kb fragment termed 47-4B. The 63-1 clone (corresponding to probes 9-14[5]) was also subcloned by EcoRI/*Hin*cII digestion as described previously.[14]

The human DMD cDNA subclones 47-4B and 44-1 were also used to screen 2 human genomic libraries as described previously.[15] Genomic clones containing 8 separate exons were isolated and used to determine the exon/intron structure of this portion of the DMD gene. Exon-containing restriction fragments were subcloned from each isolate and sequenced either manually or with an Applied Biosystems Model 370A Automated DNA Sequencer using vector- and insert-specific synthetic oligonucleotide primers as described previously.[15] The primers were prepared with an Applied Biosystems model 340A DNA synthesizer. Exon/intron boundaries for the 4.1 and 0.5 kb *Hin*dIII restriction fragment exons detectable with probe 47-4B, as well as the 1.2/3.8 kb *Hin*dIII restriction fragment exon detectable with probe 44-1, have been reported elsewhere.[15]

Results. Of the 160 affected males analyzed in these studies, approximately 90% did not show gene deletions when screened with genomic probes for the DMD gene. Using the human cDNAs, we further analyzed these individuals for the presence of genetic alterations. A sample autoradiograph displaying the typical hybridization pattern resulting from Southern blot analysis with each of the cDNA subclones is shown in figure 1. The affected male in this figure does not show a detectable deletion in the DMD gene. Partial gene deletions were detected in 56% of the affected males studied using the cDNAs shown in this figure.

The deletions detected using the human cDNAs were distributed unevenly throughout the DMD locus. Figure 2 displays the distribution of the 5′ and 3′ breakpoints for those deletions in which the endpoints were defined. The vast majority of deletions encompassed 2 high-frequency deletion regions (HFDRs) in the proximal and distal segments of the gene. Based on the long-range physical map of the gene,[16] the proximal region is centered approximately 500 kb from the 5′ end of the gene and the distal region is centrally located approximately 1,200 kb from exon 1. Within both these regions, there is a preponderance of deletion breakpoints within a limited number of introns. However, for those deletions that have 1 breakpoint within these specific introns, the 2nd breakpoints are distributed in a more heterogenous manner (see below, figure 2).

Sequence information identifying the first 21 exon/intron boundaries has recently been published.[17,18] We have applied this information to deletions in the proximal HFDR in order to test the hypothesis that the primary determinant of the relative severity of the dystrophy is the effect that a deletion exerts upon the resultant mRNA translational reading frame.[19] Twelve of the deletions we detected in the proximal HFDR had both ends of the deletions located within the region of the gene containing the first 21 exons (figure 3A). Seven of these 24 breakpoints (29%) were localized to the intron between the 4.6 and 7.5 kb *Hin*dIII fragments (exons 7 and 8). Ten of the deletions were associated with a defined disease phenotype based upon patient medical records and the clinical data presented in table 1. Three affected males (109, 474, and 483) were too young for DMD or BMD classification and were therefore not categorized for this study. The number following each deletion displayed in figure 3A indicates the predicted effect a deletion would exert on the translational reading frame of the resultant dystrophin mRNA assuming precise joining of the exons flanking a deletion. Examples of predictions made for 2 types of deletions in this region are shown in figure 4. Both the deletions shown in this figure are predicted to disrupt

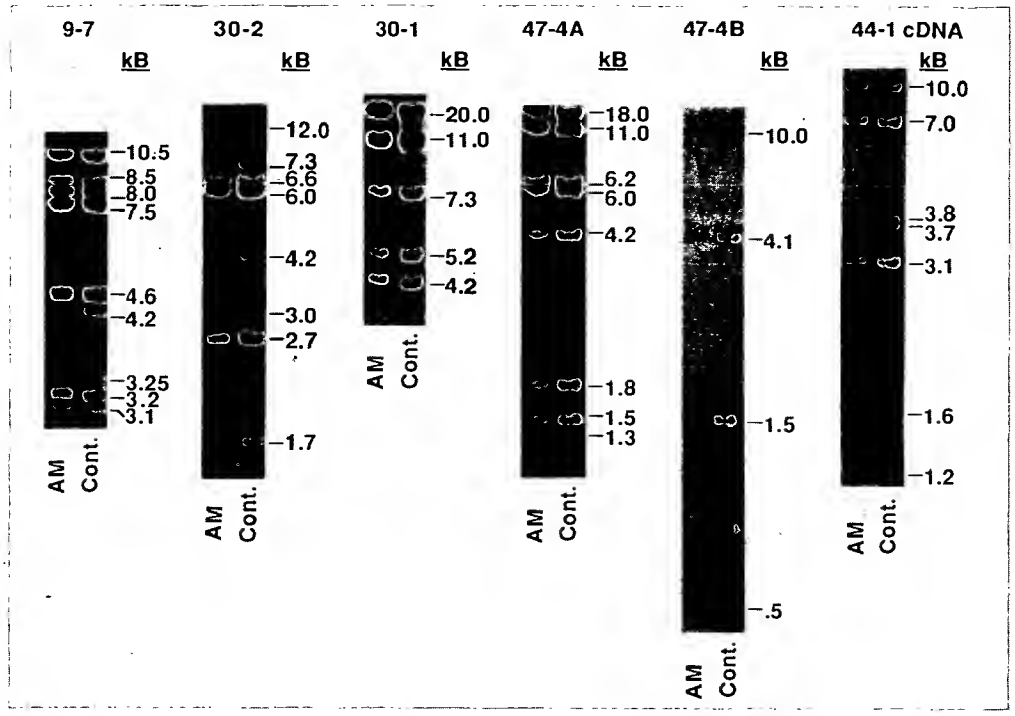

Figure 1. Southern analysis using human DMD cDNAs. Representative autoradiographs displaying the normal pattern of hybridization obtained with HindIII-digested human DNA following probing with the indicated cDNAs. AM = affected male; Cont = control female. In these examples, the affected male did not have a detectable deletion in the DMD locus using the cDNA probes.

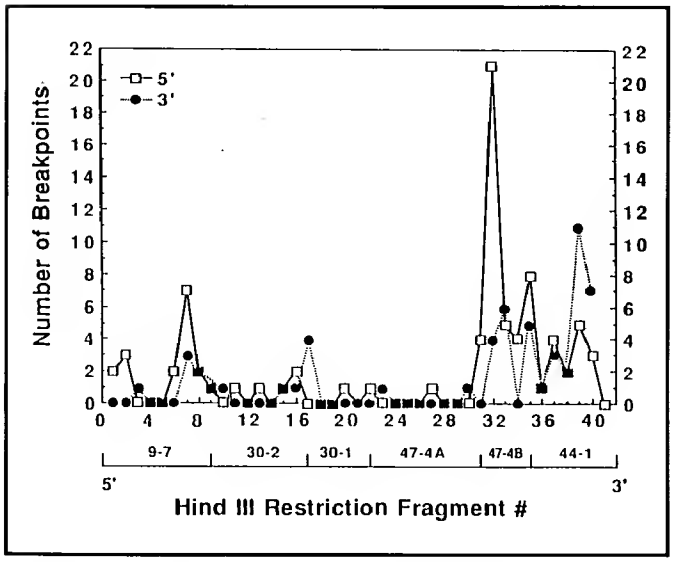

Figure 2. Distribution of deletion breakpoints in the DMD locus. A graphic summary of the number of 5′ and 3′ deletion breakpoints detected per exon-containing HindIII restriction fragment number from 5′ to 3′ (left to right) across the DMD locus. The 5′ and 3′ breakpoints are indicated as shown in the figure; points of overlap are indicated by filled squares. The cDNA probes used to detect these deletions are shown in corresponding 5′ and 3′ order along the bottom of the plot. Approx. 22% of the 3′ breakpoints were located in the 63-1 portion of the gene (not shown).

the translational reading frame of the dystrophin mRNA; however, the deletions of exons 3-7 (cases 237 and 469) are associated with an intermediate BMD/DMD phenotype. An autoradiograph that displays the 4.2-4.6 kb *Hin*dIII fragment deletion (exons 3-7) for case 469 is shown in figure 3B. These two unusual cases will be discussed in greater detail later in this paper. A similar analysis for the remaining deletions in this region supports the general hypothesis that a deletion that leads to production of a frame-shifted dystrophin mRNA is associated with DMD. This conclusion is supported by the observation that even small frame-shift-producing deletions, such as those seen in cases 158 and 152 that involve a single exon, are associated with severe DMD. However, cases 237 and 469 may serve as exceptions to this general rule (figure 4) and suggest that alternative mechanisms exist to compensate for the production of what would otherwise be a severely truncated dystrophin molecule.

Approximately 70% of the deletions were detected with the 47-4B and 44-1 cDNAs and were localized to the central HFDR. We have observed that 21 of the 59 deletions in this region had their 5′ breakpoints in the intron between the 4.1 and 0.5 kb *Hin*dIII fragments (figure 2). Therefore, 36% of deletions in this region had a 5′ breakpoint localized to this one intron. Table 2 presents correlations between clinical and molecular data for 26 patients whose deletions were contained within this region and for whom a differential diagnosis was made. We have recently isolated genomic clones containing exons from this region of the DMD gene, which has allowed us to determine the exon/intron boundaries for 8 consecutive exons from the central HFDR (table 3). This information has allowed us to predict the dystrophin translational reading frame resulting from the 40 deletions whose boundaries are contained within the region (figure 5A). The sequence of 3 of the clones[15] allowed us to refine the order of the exon-

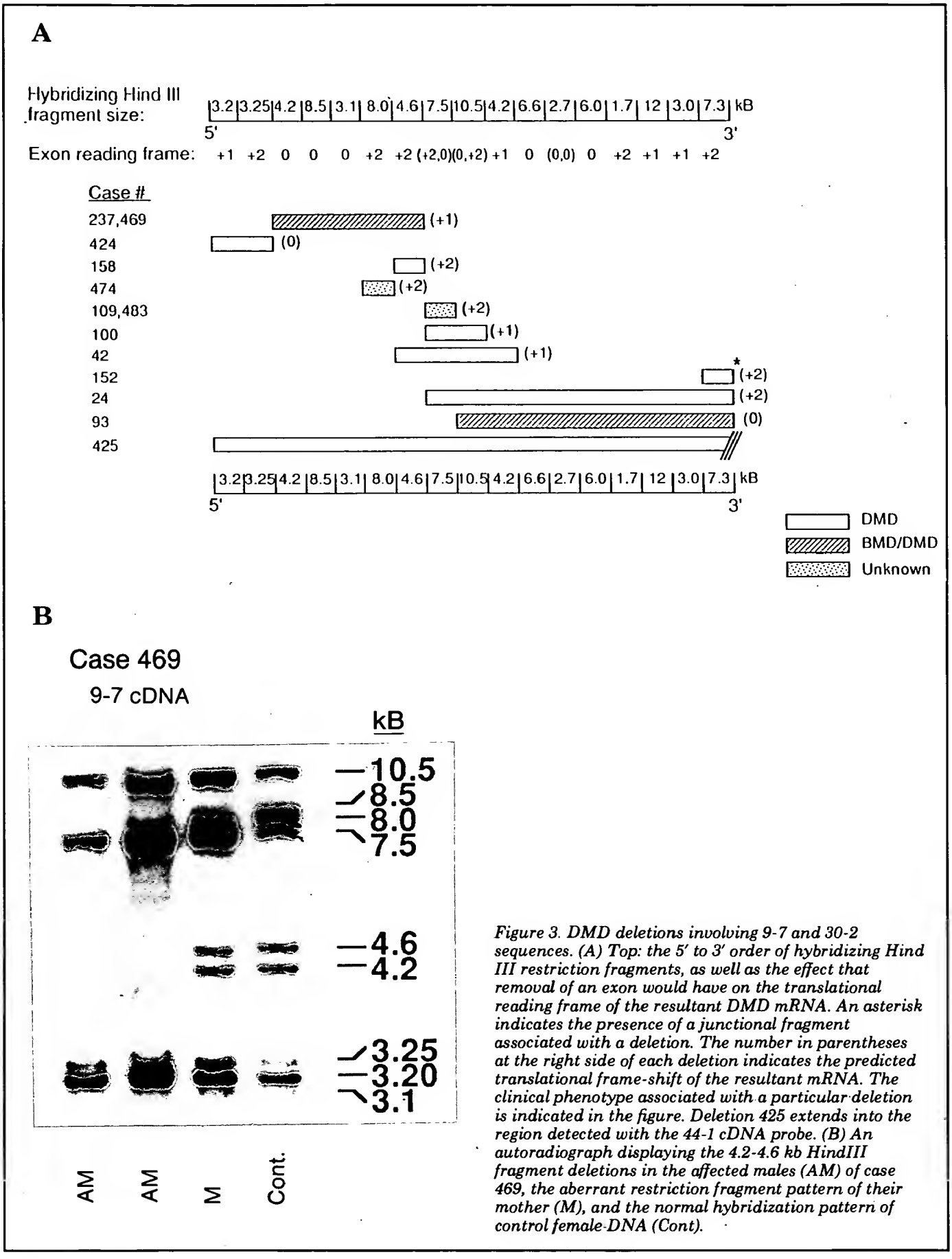

Figure 3. DMD deletions involving 9-7 and 30-2 sequences. (A) Top: the 5′ to 3′ order of hybridizing Hind III restriction fragments, as well as the effect that removal of an exon would have on the translational reading frame of the resultant DMD mRNA. An asterisk indicates the presence of a junctional fragment associated with a deletion. The number in parentheses at the right side of each deletion indicates the predicted translational frame-shift of the resultant mRNA. The clinical phenotype associated with a particular deletion is indicated in the figure. Deletion 425 extends into the region detected with the 44-1 cDNA probe. (B) An autoradiograph displaying the 4.2-4.6 kb HindIII fragment deletions in the affected males (AM) of case 469, the aberrant restriction fragment pattern of their mother (M), and the normal hybridization pattern of control female-DNA (Cont).

Table 1. Molecular and clinical data correlations for 5′ HFDR deletions

Case no.	Deleted *Hin*dIII* fragment (kb)	Frame-shift†	Clinical data‡ DX	WC	Age	Phenotype§
237	4.2-4.6	Yes	6	N/A	10	BMD/DMD
469	4.2-4.6	Yes	3 3	15 17	31 35	BMD/DMD
424	3.2-3.25	—	6	10	12	DMD
158	4.6	Yes	7	8	20	DMD
474	8.0	Yes	5	N/A	11	Unknown
109	7.5	Yes	6		7	Unknown
483	7.5	Yes	6.5		9	Unknown
100	7.5, 10.5	Yes	1.5	8	24	DMD
42	4.6-4.2	Yes	5	10	13	DMD
152	7.3/JNCT.	Yes	4.5	8	13	DMD
24	7.5-7.3	Yes	3	9	20	DMD
93	10.5-7.3	No	5.5	N/A	13.5	BMD/DMD
425	All	—	7	13	18	DMD

* Size of exon-containing *Hin*dIII restriction fragments (in kb) which are deleted, in 5′ to 3′ order, determined by Southern analysis with the 9-7 and 30-2 cDNAs. "JNCT." refers to a junctional fragment detected in association with a deletion.
† Predicted effect that a deletion would have upon dystrophin mRNA translation. "Yes" refers to a deletion that would disrupt an open translational reading frame, causing a change in translation. "No" refers to a deletion that removes in-frame exons, such that an open reading frame is preserved. "—" indicates that exon 1 is deleted and mRNA is therefore not expected to be produced.
‡ Ages of affected males (in years), as indicated at time of diagnosis (DX), wheelchair-dependence (WC), and at the present time (age). "N/A" refers to a patient who is at least 10 years of age and is not wheelchair-dependent.
§ Disease phenotype based upon clinical data presented in the table and patient medical records. "DMD" indicates classic Duchenne, "BMD/DMD" indicates an intermediate case of Becker/Duchenne, and "Unknown" indicates that the affected male was too young to make a differential diagnosis.

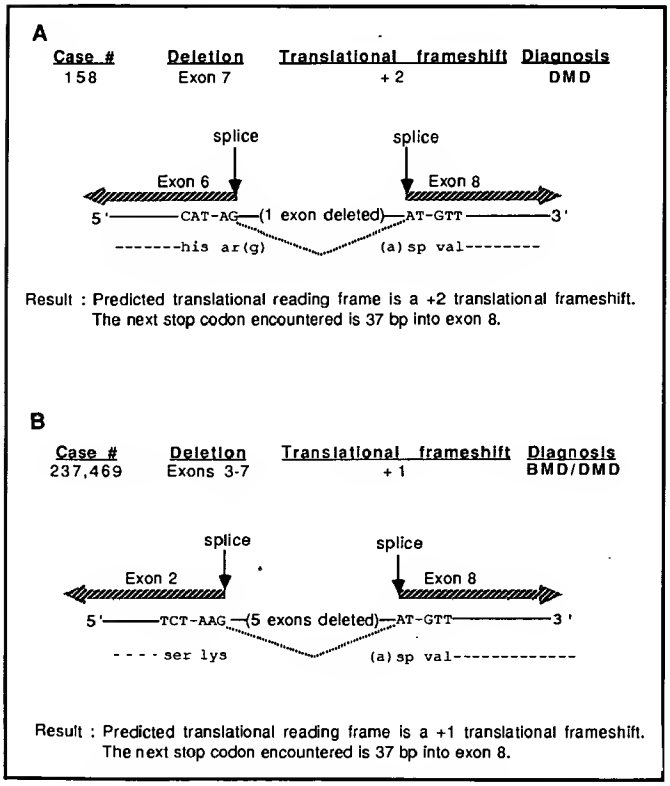

Figure 4. Predictions of effects of gene deletions in the proximal HFDR. Examples of predictions on mRNA structure resulting from 5′ DMD gene deletions that should result in a translational frame-shift of the resultant dystrophin mRNA. Displayed in the figure are the exons flanking a deletion as well as the bases that define the exon/intron boundaries and the corresponding amino acids.

containing *Hin*dIII fragments (see figure 5A) initially reported by Koenig et al.[5] Figure 5B shows the results of Southern analysis for 2 of the unusual deletions observed in this region, whereas figure 6 displays examples of predictions of mRNA splicing patterns for several of the deletions based upon the exon sequence data shown in table 3.

Several conclusions may be drawn from correlations between clinical data and the predicted effects of deletions in this region on dystrophin mRNA translation. First, a large deletion removing multiple exons does not necessarily result in a more severe disease phenotype. Case 156 clearly displays a deletion of more exons than cases 70, 204, 383, 521, or 130, yet has the mildest phenotype (BMD) of any of these patients (figure 6A and table 2). Second, apparently identical deletions as detected by Southern analysis can be associated with markedly different disease phenotypes. A deletion of the 4.1 kb *Hin*dIII fragment exon is associated with DMD in 2 cases and an intermediate disease phenotype in another case (figures 5B, 6B, and table 2). Similar results are apparent with cases 209 and 420, as well as cases 523, 546, and 22 (figure 5A and table 2). Third, although there is a general positive correlation between disease phenotypes and the predicted effects that deletions produce upon dystrophin translation, there are also clear exceptions (figure 5, A and B, and figure 6). For example, the deletion detected in case 22 should result in a deletion that interrupts the translational reading frame and thus produces a truncated dystrophin molecule, yet this affected male presents a typical BMD phenotype (figure 6C and table 2). These results clearly illustrate that deletions in both the 5′ and central coding regions

Table 2. Molecular and clinical data correlations for central HFDR deletions

Case no.	Deleted *Hin*dIII* fragment (kb)	Frame-shift†	Clinical data‡ DX	WC	Age	Phenotype§
113	0.5-3.7	Yes	5	9	13	DMD
183	0.5-3.7	Yes	6	8	15 (D)	DMD
310	0.5-3.7	Yes	—	7	—	DMD
130	1.5-10	Yes	5	8	9	DMD
156	0.5-1.6	No	9	21	26	BMD
135	1.2-1.6	No	7	N/A	15	BMD
70	1.2-3.7	Yes	3		7	DMD (F)
204	1.2-3.7	Yes	5	9	13	DMD
383	1.2-3.7	Yes	5	10.5	19	DMD
521	1.2-3.7	Yes	4	11	16	DMD (F)
531	10-3.7	Yes	1.5		1.5	DMD (F)
38	4.1	Yes	6	—	17 (D)	DMD
413	4.1	Yes	6	8	11.5	DMD
523	0.5	Yes	—	13	17	DMD
546	0.5	Yes	3 4		8 5	DMD (F)
201	3.8-3.7/JNCT.	Yes	6.5	11	20	DMD
423	1.6-3.7	Yes	4	—	14 (D)	DMD
132	3.1	Yes	5	13	20	DMD
168	3.1	Yes	7	15	27 (D)	DMD
471	3.1	Yes	2	10	22	DMD
542	3.1	Yes	6	15	18	DMD
420	3.7	Yes	2	10	17	DMD
209	3.7	Yes	5	18	23	BMD/DMD
361	4.1	Yes	4	10	32	BMD/DMD
22	0.5	Yes	10	19	34	BMD

* Size of exon-containing *Hin*dIII restriction fragments (in kb) that are deleted, in 5′ to 3′ order, as determined by Southern analysis with the 47-4B and 44-1 cDNAs. "JNCT." refers to a junctional fragment detected in association with a deletion.
† Predicted effect that a deletion would have upon dystrophin mRNA translation. "Yes" refers to a deletion that would disrupt an open translational reading frame. "No" refers to a deletion that removes in-frame exons, such that the open reading frame is preserved.
‡ Ages of affected males (in years), as indicated at time of diagnosis (DX), wheelchair-dependence (WC), and at the present time (age) or at death (D). "N/A" refers to a patient who is at least 10 years of age and is not wheelchair-dependent. "—" indicates unavailable information.
§ Disease phenotype based upon clinical data presented in the table and patient medical records. "DMD" indicates classic Duchenne, "BMD/DMD" indicates an intermediate case of Becker/Duchenne, "BMD" indicates classic Becker, and "DMD (F)" indicates a familial case of DMD in which another affected male has been identified.

Table 3. Exon structure of the central HFDR of the DMD gene

	Exon boundaries*	Size†	Frame‡	*Hin*dIII fragment§
A.	5′-GCGATTTGAC .....GTATCTTAAG-3′	148 bp	+1	4.1 kb
B.	5′-GAACTCCAGG .....GAAAAAAGAG-3′	176 bp	+2	0.5 kb
C.	5′-GCTAGAAGAA .....GCAAGTCAAG-3′	148 bp	+1	1.5 kb
D.	5′-TTACTGGTGG .....GTGGATAAAG-3′	150 bp	0	10 kb
E.	5′-GTTTCCAGAG .....TGACGTTAAG-3′	186 bp	0	1.2/3.8 kb
F.	5′-GAAACTGAAA .....GCCAGTGAAG-3′	102 bp	0	1.6 kb
G.	5′-AGGAAGTTAG .....ATTGGAGCCT-3′	109 bp	+1	3.7 kb
H.	5′-CTCCTACTCA .....CAAGCAGAAG-3′	233 bp	+2	3.1 kb

* The first and last 10 base pairs of each exon are indicated. The exons A-H are listed in 5′-3′ order along the DMD gene.
† The size in base pairs of each exon.
‡ The DMD mRNA translational reading frame-shift that would result from each exon being deleted from the transcript.
§ The size in kilobase pairs of the genomic *Hin*dIII restriction fragment that each exon is located in. Exon "E" contains an internal *Hin*dIII restriction endonuclease recognition sequence and therefore overlaps 2 such fragments.

of the gene do not always result in phenotypes that correlate with predicted dystrophin mRNA reading frames.

Discussion. The results reported here describe a detailed analysis of deletions occurring in Duchenne and Becker muscular dystrophy patients as well as patients who exhibit an intermediate disease phenotype. Using human cDNAs for the DMD locus, we have detected deletions in 56% of the affected males studied, a value that is similar to the 50% to 67% values recently reported by other laboratories.[5-8,20] The combined use of the 4 probes 9-7, 30-2, 47-4B, and 44-1 detected 98% of these deletions. Prior to the use of cDNA clones, our

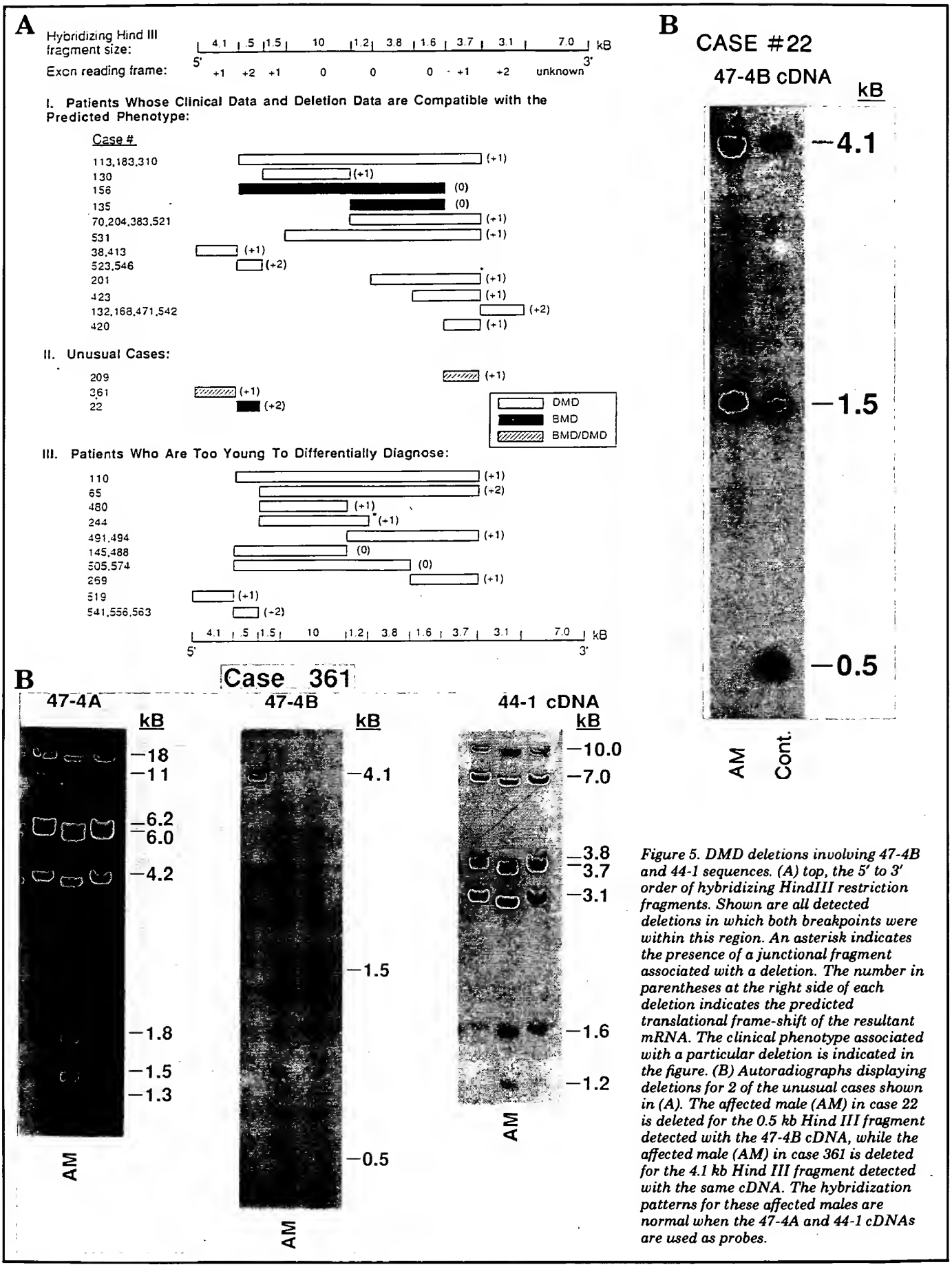

Figure 5. DMD deletions involving 47-4B and 44-1 sequences. (A) top, the 5′ to 3′ order of hybridizing HindIII restriction fragments. Shown are all detected deletions in which both breakpoints were within this region. An asterisk indicates the presence of a junctional fragment associated with a deletion. The number in parentheses at the right side of each deletion indicates the predicted translational frame-shift of the resultant mRNA. The clinical phenotype associated with a particular deletion is indicated in the figure. (B) Autoradiographs displaying deletions for 2 of the unusual cases shown in (A). The affected male (AM) in case 22 is deleted for the 0.5 kb Hind III fragment detected with the 47-4B cDNA, while the affected male (AM) in case 361 is deleted for the 4.1 kb Hind III fragment detected with the same cDNA. The hybridization patterns for these affected males are normal when the 47-4A and 44-1 cDNAs are used as probes.

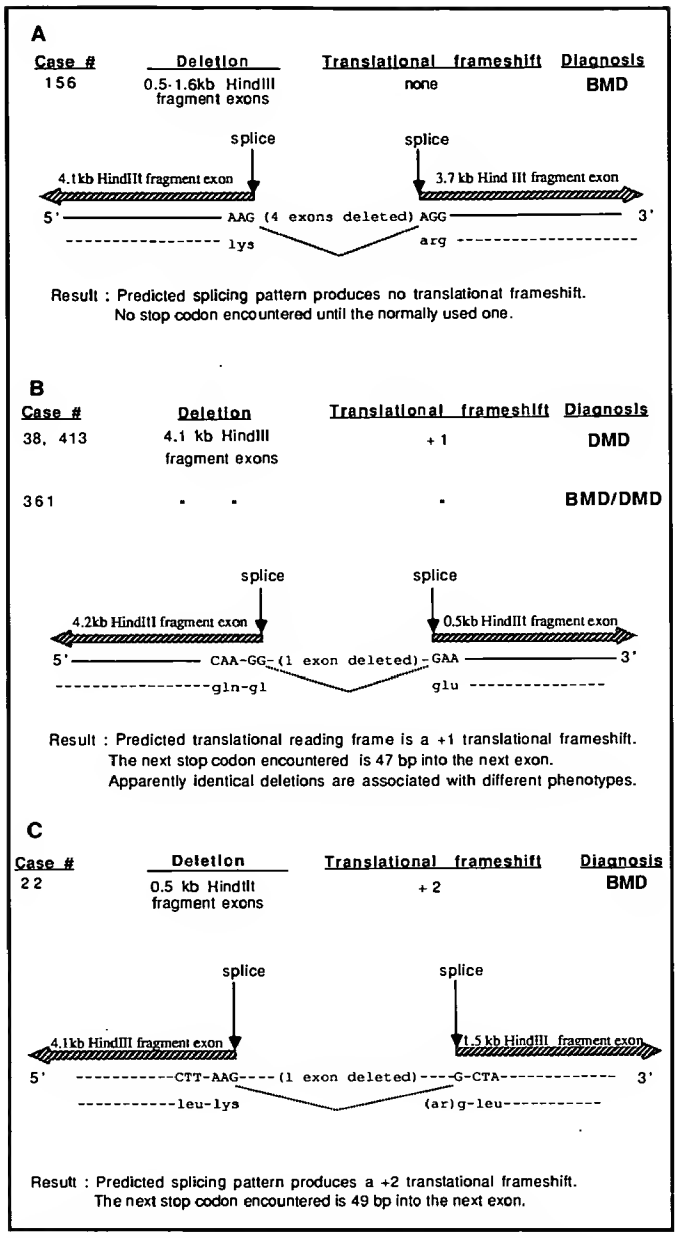

Figure 6. Predictions of effects of gene deletions in the distal HFDR. Examples of predictions made on dystrophin mRNA structure for central DMD gene deletions that should result in a translational frame-shift of the resultant dystrophin mRNA (B and C) and a deletion that should maintain the open reading frame of the resultant dystrophin mRNA (A). Displayed in the figure are the exons flanking a deletion as well as the bases that define the exon/intron boundaries and the corresponding amino acids.

laboratory,[11,21] as well as others,[22] had detected a deletion frequency of approximately 10% in affected males by using a limited number of genomic probes. The availability of cDNAs for the DMD gene has allowed a more precise examination of genomic rearrangements involving exon sequences within the locus of affected individuals. At least 5% of affected males display partial endoduplications of the DMD gene (L.L.B., unpublished observations). The remaining 40% of mutations are suspected to be the result of microdeletions or point mutations, which are generally not detectable by Southern analysis. Molecular identification of these latter types of mutations will require more sophisticated technology, such as that developed for point mutation detection in the HPRT gene.[23] However, the immense difference in size of HPRT (1,650 bp) and dystrophin (14,000 bp) mRNAs may require further refinement of techniques for point mutation detection.

Although deletions are heterogeneously distributed throughout the DMD locus there appear to be certain regions of the gene, which we have termed "HFDRs," that are frequently included in deletions. Within these regions there is a preponderance of deletion breakpoints within specific introns. These include the intron between the 4.6 and 7.5 kb *Hin*dIII restriction fragments detected with the 9-7 cDNA subclone and between the 4.1 and 0.5 kb *Hin*dIII restriction fragments detected with the 47-4B cDNA subclone. Of those deletions with 1 breakpoint within these specific introns, the site of the other breakpoint is considerably heterogeneous. This observation may implicate the existence of specific sequences that cause these regions to be highly mutable, and further DNA sequence analysis will be required to explore this possibility. Of interest is the apparent paucity of deletions in the 3′ regions of the gene corresponding to the 63-1 cDNA subclones. The only cases we have confirmed to be deleted for the 3′ end of the DMD gene are patients with complex glycerol kinase deficiency.[14] A retrospective examination of deletions associated with different clinical manifestations of DMD/BMD resulted in a lack of correlation between the general location and size of deletions and the clinical severity of the disease. We have observed that similar deletions within the proximal and distal regions of the gene are associated with different disease phenotypes and that very large deletions within the gene do not necessarily result in more severe forms of muscular dystrophy. Similar conclusions were reached by Lindlöf et al[24] after comparing 37 DMD and 11 BMD patients.

Having established these observations, we investigated the recent suggestion by Monaco et al[19] that the clinical severity of the dystrophy is determined primarily by the effect that an intragenic deletion would have upon the translational reading frame of a DMD mRNA, rather than the size of the deletion. A comparison of deletion cases at the 5′ end of the DMD gene was made for those deletions where both endpoints mapped within regions of known gene structure[17,18] (figure 3A). Most of the deletions in this region were associated with the DMD phenotype (table 1), and were also predicted to disrupt the translational reading frame of the dystrophin mRNA. However, cases 469 and 237 serve to illustrate that prognostications of the clinical severity of DMD/BMD should not be based entirely upon the predicted effect on mRNA translation resulting from a deletion. The two brothers in case 469 display an intermediate case of BMD/DMD; these brothers were diagnosed at an early age but were not wheelchair-dependent until their late teens. Both are still alive in their early to mid 30s, but are currently severely affected. The affected male in case 237 also possesses a mild form of dystrophy, and although he is

too young to be certain of a differential DMD/BMD diagnosis, the clinical progression of his disease is less than other affected males of the same age who possess small deletions in this region, which are predicted to result in DMD (cases 158, 100, and 152; table 1). The three males in cases 469 and 237 all display a similar deletion of the same 4.2-4.6 kb *Hin*dIII restriction fragments detected with the 9-7 cDNA. This deletion might be expected to produce a DMD phenotype, since removal of the 5 exons within the deleted region would be predicted to produce an mRNA containing an in-frame translational stop codon in exon 8 (figure 4B and reference 17). The 7.5 kb *Hin*dIII restriction fragment flanking the 3′ end of the deletion contains 2 exons,[17,18] and although this fragment appears normal on Southern blots, an mRNA with an open reading frame could result if the 5′ exon was deleted such that a deletion junction fragment of approximately normal size was detected with the cDNA. However, polymerase chain reaction amplification of the 5′ exon from the 7.5 kb *Hin*dIII fragment has revealed this exon and its flanking introns to be intact, a finding indicating that the deletion should disrupt the DMD mRNA open reading frame (reference 15 and J.S.C., unpublished observation). These findings are corroborated by Malhotra et al,[18] who described 6 BMD and 5 BMD/DMD patients who all possessed similiar 4.2-4.6 kb *Hin*dIII fragment deletions. Since this apparent frame-shift lesion occurs at the extreme 5′ end of the gene, the mild DMD phenotype could perhaps be explained by the occurrence of an additional mutation that might restore the mRNA reading frame. Translational reinitiation has also been postulated as a potential mechanism for producing a functional dystrophin molecule from a gene containing the 4.2-4.6 kb *Hin*dIII fragment deletion.[18] Alternatively, differential splicing of the mRNA could correct for a mutation that would be predicted to result in a translational frame-shift. Examples of mutation-induced alternative splicing mechanisms, such as exon skipping and cryptic splice site utilization, have recently been noted in other genetic diseases such as retinoblastoma,[25] β-thalessemia,[26] and Lesch-Nyhan syndrome.[27] While the presence of a second promoter might explain unusual phenotypes resulting from deletions at the 5′ end of the gene,[18] such a mechanism is probably not operative toward the 3′ end of the locus. Sequence analysis of these unusual patients' dystrophin mRNA will be required to resolve this question.

Using sequence data obtained from genomic clones isolated with the 47-4B and 44-1 cDNAs, we were able to extend these previous observations on a limited number of different deletions at the 5′ end of the gene to the central HFDR that was involved in 69% of the DMD gene deletions identified in this study. Figure 5A illustrates those deletions whose breakpoints fall in the region of gene structure we have determined. BMD cases 135 and 156 each resulted from the deletion of exons that would not disrupt the mRNA reading frame. Case 201 (DMD) is of interest because it displayed a deletion that encompassed one, but not both, of the 1.2 and 3.8 kb *Hin*dIII restriction fragments (figure 5A). These restriction fragments overlap the same exon[15] and this patient displayed a deletion junction fragment (table 2), observations suggesting that the deletion ends within or very close to this exon. If the exons were intact, patient 201 would possess an out-of-frame deletion, in agreement with the diagnosis of DMD (table 2).

Although for most of the DMD cases in this region a positive correlation existed between predicted phenotype (based on a deletion's effect on mRNA translation) and the clinical phenotype, there were clear exceptions to this rule. For example, cases 209, 361, and 22 are associated with deletions that should disrupt dystrophin translation, resulting in a truncated protein and DMD (figure 5A and table 2). Instead, these cases are classified as intermediate or BMD. In addition to these findings, we have observed cases such as 38, 413, and 361, in the central HFDR, that display apparently identical deletions but are associated with different disease phenotypes (figure 5, A and B, and table 2).

Monaco et al recently reported[19] that BMD patients bearing similarly large deletions within the 5′ portion of the DMD locus presented somewhat variable clinical phenotypes. An analogous observation was also noted by Forrest et al,[7] who reported that an apparently identical deletion was associated with markedly different disease phenotypes in affected individuals within a familial case of BMD. These results suggested that factors in addition to the deletion of a specific exon are important in determining the disease phenotype within a family. Our observations, combined with those of Monaco et al[19] and Forest et al,[7] suggest that deletions that remove the same exons can be associated with different disease phenotypes. Furthermore, we find that predictions of disease severity based on the expected effects on dystrophin translation are not always accurate.

In conclusion, our results demonstrate that multiple deletions in various regions of the DMD gene can produce disease phenotypes that are not predictable by Southern analysis and knowledge of the exon/intron structure of the gene. The observation that such deletions exist in the 5′ and central HFDRs suggests that multiple mechanisms may be involved in producing these unpredictable phenotypes. Although the predicted maintenance of an open reading frame for dystrophin translation is often associated with a less severe form of muscular dystrophy, it is not entirely indicative of the clinical progression of milder forms of the disease. In particular, the possibility that a deletion could trigger the utilization of cryptic splice sites indicates that prognostications of the clinical severity of DMD/BMD should *not* be based entirely upon the predicted effect that an intragenic deletion would have on dystrophin mRNA translation. The continued isolation and identification of exon-containing genomic clones for the DMD gene will allow for the determination of the precise exon sequences missing from all DMD- or BMD-producing deletions. Detailed comparisons of deletions leading to the different disease phenotypes arising from the DMD locus, as well as the mRNAs produced from these mutant genes, should provide in-

sight into the functional domains of dystrophin and the molecular mechanisms involved in disease formation.

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#37

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Ser. No.:	08/315,673	§	Docket No. D-5050-C3
Filing Date:	09/30/94	§	Examiner: A. Marschel
Applicant:	Caskey, et al.	§	Art Unit: 1807
Title:	Multiplex Genomic DNA Amplification for Deletion Detection	§	

Commissioner of Patents and Trademarks
Washington, D.C. 20231

DECLARATION OF UMADEVI TANTRAVAHI UNDER 37 C.F.R § 1.132

Dear Sir:

I, Umadevi Tantravahi, do hereby depose and say as follows:

1. I am the Director of Molecular Genetics and Cytogenetics in the Department of Pathology and Laboratory Medicine at Women & Infants Hospital of Rhode Island, located in Providence, Rhode Island. I am skilled in the area of molecular biology and DNA amplification. My *curriculum vitae* is attached.

2. Multiplex DNA amplification, using the methods of Caskey et al., has been performed successfully in my laboratory.

3. Prior to the development of the technology by Dr. Caskey's group, I had not had any experience with multiplex reactions.

4. Using Dr. Caskey's technique, I and other members of my laboratory group have multiplexed other loci.

5. I hereby declare that all statements herein of my own knowledge are true, that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 12/27/94

Umadevi Tantravahi

Umadevi Tantravahi

#37

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DECLARATION OF ANDRES METSPALU UNDER 37 C.F.R § 1.132

Dear Sir:

I, Andres Metspalu, do hereby depose and say as follows:

1. I am a Professor at the Institute of Molecular and Cell Biology at Tartu University in Tartu, Estonia. I am skilled in the area of molecular biology and DNA amplification. My *curriculum vitae* is attached.

2. Using the protocol and materials provided by Dr. Caskey in 1992 and 1993, multiplex amplification was performed successfully in my laboratory. Dr. Caskey made DMD multiplex PCR diagnostic kits available to my laboratory. The multiplex technique has proved to be very useful and established DMD molecular diagnostics at Tartu University. The kits have been found to be stable for more than two years and give reproducible results.

3. Prior to being provided with the methods and materials by Dr. Caskey, I had not performed multiplex reactions involving more than two sets of primers.

4. There was a need in the art for multiplex amplification using more than two sets of primers. Previously, screening for a plurality of DNA sequences required a large number of separate assays for each sequence. The multiplex assay of Caskey et al. thus saves time and money.

5. The positive results with DMD kits, in combination with our skill in the art, have led my colleagues and I to design other multiplex PCR tests. We designed our own multiplex PCR tests for the CFTR and PAH genes.

6. I hereby declare that all statements herein of my own knowledge are true, that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: January 24, 1995

Andres Metspalu

Curriculum Vitae

Andres Metspalu
4-33 Kaunase Str.
Tartu, Estonia, EE2400

Education

M.D. Tartu University, June 1976

Ph.D. in molecular buiology 1980.Tartu University Structure and function of the eukaryotic ribosome. Scientific adviser Prof. Artur Lind. Title of the thesis "Interaction of 5S RNA, 5.8S RNA and tRNA with rat-liver ribosomal proteins"

Postdoctoral research:

1. Columbia University, New York, USA, Prof. Alex Tzagaloff laboratory studies on mitDNA and Yale University, New Haven, USA, Prof. Joan Steitz laboratory, studies on snRNPs. 1981-1982.

Employment

1. 1976-1984 Junior and senior scientist at Laboratory of Molecular Biology at Tartu University
2. 1985. 1992 Head of the Laboratory of Gene Expression at Tartu University.
3. 1986-1992 Research Director of the Estonian Biocenter.
4. July 1992 - present. professor of Biotechnology at Tartu University.

Honors and Awards

Estonian State Prize for science.
FEBS short term fellowship in 1985
EMBO short term fellowship in 1988
DAAD fellowship in 1991
EEC fellowship in 1993
ISF grant in 1994

Professional Societies

European Society of Human Genetics

Selected Publications

1. Metspalu A., M. Saarma, R. Villems, M. Ustav, A. Lind. Interaction of 5S RNA, 5,8S RNA and tRNA with rat-liver ribosomal proteins. *Eur. J. Biochem.* 91: 73-81, 1978.

2. Dente L., M.-G. Pizza, A. Metspalu, R. Cortese. Structure and expression of the genes coding for human a_1-acid glycoprotein. *The EMBO J.* 6: 2289-2296, 1987.

3. Steitz J., C. Berg, J. P. Hendrick, H. La Brance-Chabot, A. Metspalu, J. Rinke, T. Yario. A 5S RNA/L5 complex is a precursor to ribosome assembly in mammallian cells. *J. Cell Biol.* 106: 545-546, 1988.

4. Metspalu A., A. Rebane, S. Hoth, M. Pooga, J. Stahl and J. Kruppa. Human ribosomal protein S3: Cloning of the cDNA and primary structure of the protein. *Gene* 119: 313-316, 1992.

5. Pata I., S. Hoth, J. Kruppa, a. Metspalu. The human ribosomal protein S6 gene: Isolation, primary structure and location in chromosome 9. *Gene* 121:387-392, 1992.

6. Pata, I and A.Metspalu. A dinucleotide repeat polymorphism at the ribosomal protein S6 (RPS6) gene. Human Molec. Genetics, 1993, 2, 1749.

7. Shumaker J.M., A.Metspalu and C.T. Caskey. Sequencing by Solid Phase Primer Extension. (submitted for publication).

#37

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Washington, D.C. 20231

DECLARATION OF HUGO A. BARRERA-SALDANA UNDER 37 C.F.R § 1.132

Dear Sir:

I, Hugo A. Barrera-Saldaña, do hereby depose and say as follows:

1. I am the Chair of the Department of Biochemistry at Universidad Autonoma de Nuevo Leon in Monterrey, Mexico. I am skilled in the area of molecular biology and DNA amplification. My *curriculum vitae* is attached.

2. Using the protocol and materials provided by Dr. Caskey, multiplex amplification was performed successfully in my laboratory.

3. Prior to being provided with the methods and materials by Dr. Caskey, I had not performed multiplex reactions involving more than two sets of primers.

4. There was a need in the art for multiplex amplification using more than two sets of primers. Previously, screening for a plurality of DNA sequences required a large number of separate assays for each sequence. The multiplex assay of Caskey et al. thus saves time and money.

5. My experience with Dr. Caskey's multiplex technique has led me to develop multiplex PCRs for other genes.

6. I hereby declare that all statements herein of my own knowledge are true, that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: January 20, 1995

Hugo A. Barrera-Saldaña

CURRICULUM VITAE

Professor Hugo A. Barrera Saldaña, Chairman
Department of Biochemistry
Faculty of Medicine,
Autonomous University of Nuevo Leon (AUNL)

BIODATA:

Born in Miguel Alemán, Tamaulipas, México. (October 7, 1957). Married to Sonia Barrera; two children, Andrea and Daniela.

Home Address: Miguel de Cervantes Saavedra # 825. Colinas de San Jerónimo, Monterrey, N L. México 64630. Tel. (528) 346-5798.

Work address: Departamento de Bioquimíca, Facultad de Medicina de la Universidad Autonoma de Nuevo León. Apartado Postal 3-4125 Monterrey, N.L., México 64460. Tel. (528) 329-4050 Ext. 2580 and 4173. Fax. (528) 333-7747. E-mail: hbarrera@ccr.dsi.uanl.mx

EDUCATION:

- B.S. in Biology (March,1979), magna cum laude, AUNL
- Ph.D.(December,1982) University of Texas Health Science Center-Houston
- Postdoctorate (1983-19984) with Professor Pierre Chambon at LGME-CNRS. in Strasbourg, France.

NATIONAL AWARDS:

- AUNL Research in Basic Sciences '(1984,1985,1986 and 1990)
- National Food Research (1987).
- Creative, Scientific and Technological Projects for Young Researchers (1988).
- "Dr. George Rosenkranz"-Syntex (1989).
- Human Genetics Congresses (1989, First place;1991, second place; 1992, third place and best poster).
- Biomedical Research Encounters (twelve prices between 1990-1993).
- Congresses of Internal Medicine (1990, second place 1994, first place).
- "Guillermo González Camarena"-Science and Technology Award for Young Researchers (1990, biotechnology section).
- Nuevo Leon State-Texas Branch of American Society of Microbiology Meeting (1992, best paper).
- Weizmann-National Scientific Research Academy (1992, for Best Doctoral Thesis Advisor, in the Natural Sciences)
- "CONAQUIC 93"-National Confederation of Clinical Chemists (1993)
- Best B. Sc. Thesis Advisor , AUNL (1993).
- CARPERMOR-Mexican Association of Clinical Biochemistry (1994)
- National Chamber of the Pharmaceutical Industry Award (1994, two: one in human health and other in veterinary medicine)

GRANTS:

- My laboratory has received nearly two million dollars in grants from thc following institutions:
- Mexican Secretariat of Public Education (MSPE) for research of gene fossils in thc human genomc and thc uricase gene, part onc (1984).
- MSPE for research of gene fossils in the human genome and the uricase gene, part two (1985).
- Ricardo J. Zevada Foundation for developing the Laboratory Unit of Genetic and Engineering Expression (1985).
- National Council for Technological Education (COSNET) for developing, implementing and exchange of genetic engineering techniques (1985).
- Continuation of research on presence of gene fossils in the human genome and the uricase gene from the MSPE (1986).
- National Council of Science and Technology (CONACYT) for locating functional donains of human growth hormone through cellular culture and genetic engineering techniques (1986).
- MSPE for improving genetic engineering techniques in the production of bovine growth hormone (1987).
- MSPE for locating functional domains of human growth hormone through genetic engineering and cellular cultivation techniques (1987).
- MSPE for locating functional regions of human growth hormone (1988).
- MSPE for molecular cloning of complementary DNA to RNA messenger of the human growth hormone in E. coli (1988).
- CONACYT for production of bovine growth hormone through genetic engineering, stages I and II, molecular cloning of complementary DNA to RNA messenger (1988).
- CONACYT for detection of sequences of papilloma type 16 human virus and alterations of c-myc protooncogen in human cervicouterine cancer (1988).
- General Foods Corporation of Mexico for developing animal biotechnology projects (1988).
- CONACYT for research on adipogenic activity of chimeric polypeptides among human placental lactogen growth hormones generated by genetic engineering and cell culture techniques (1989).
- UNESCO for the molecular biology of coding gene complex for human growth and placental lactogen hormones.
- MSPE for implementation of Molecular Gene Diagnosis Program in the metropolitan area of Monterrey, Nuevo Leon, Mexico (1989).
- International Business Machines Corporation (IBM de Mexico, S.A.) for Application of microcomputers in a Molecular Biology Research Laboratory(1989).
- CONACYT for research on human growth hormone isoforms(1990).
- CONACYT for research on production of recombinant growth hormones.(1990).
- National Foundation for Health (FMS) for research on the molecular diagnosis of Cystic Fibrosis.(1992).
- CONACYT for research on the molecular diagnosis of Hemophilia A.(1992).
- CONACYT for reinforcement of our Research Unit experimental infrastructure (1993).
- CONACYT for cloning and expresing human growth hormone isoforms (1994).
- CONACYT for developmental expression of human placental lactogen genes (1994).

TEACHING AND LECTURING EXPERIENCE:

a) AUNL curricular courses.

- Instructor of Cell Physiology, Faculty of Biological Sciences AUNL (1977).
- Biochemistry for medical students, Faculty of Medicine, UANL (undergraduate program).
- Molecular Cell Biology (graduate program).
- Genetic Structure and Gene Expression (graduate program).
- Basic Techniques in Genetic Engineering (graduate program).
- Oncology resident's course on updating on Human Molecular Genetics, University Hospital of the Faculty of Medicine, A.U.N.L.
- Allergy resident's course on updating on Human Molecular Genetics, University Hospital of the Faculty of Medicine, A.U.N.L.
- Internal Medicine residents' course on updating on human molecular genetics, University Hospital of the Faculty of Medicine, UANL.
- Genetic Engineering I Selectes Topics (graduate program).
- Genetic Engineering II Selected Topics (graduate program).

b) Visiting professorships.

- **1978** Guest lecturer in Cellular Biology, Graduate School of the State Teachers University, Monterrey, Mexico.
- **Summer, 1981** Guest lecturer in Molecular Genetics, Graduate School of the Faculty of the Biological Sciences (AUNL).
- **February 1982** Guest lecturer, Gene mapping by <u>in situ</u> hybridization offered jointly with Dr. Mary E. Harper (Auguron Institute). Northeast Center for Biomedical Investigations of the Mexican Institute of Social Security, Monterrey, Mexico.
- **December, 1984** Guest lecturer, "Techniques in Recombinant DNA", Center of Advanced Studies and Research of the National Polytechnical Institute, Mexico City.
- **March, 1985** Guest lecturer, "Site-Directed Mutagenesis" Research Center Institute for Biotechnology, Autonomous National University of Mexico at Cuernavaca.
- **April, 1985** Guest lecturer, graduate Molecular Biology course at Faculty of Biological Sciences, UANL.
- **August 1985-January 1986** Guest lecturer, Biochemistry, Technological Institute, Victoria, Mexico.
- **1989** Guest lecturer of master's candidates course on the molecular biology of the eukaryotes, Graduate School, Faculty of Biological Sciences (AUNL).
- **May 1992** Guest instructor in the course of Genetic Engineering granted to the teachers of the ITESM high Schools.
- **Feb. 1992** Guest instructor in the Practical Course "Polymerase Chain Reaction and its applications to the study of Human Genome" Western Unit of Biomedical Research, IMMS. Guadalajara, Mexico.
- **Since 1992** Organizer and professor of seven Latin American Molecular Biology in Medicine Workshops offered as Satelite Courses to the Annual National Biomedical Encounter. Monterrey, Mexico and of one in the Latin American Congress of Genetics (1994, Puerto Vallarta, Mexico).
- **1992 to present** Lecturer at the review course for the National Residences Exam.
- **January1993** Coordinator and professor of the Polymerase Chain Reaction on inherited deseases offered at Medical Genetics Unit of the School of Medicine at Zulia University, Maracaibo, Venezuela.

RESEARCH EXPERIENCE:

- **1978-1979** B.S. thesis fellow at the Biomedical Research Unit, Mexican Institute of Social Security. Monterrey, N.L. Project: isolation and characterization of human placental nucleic.
- **1978 Summer** Student in the laboratory of Dr. Grady F. Saunders at the M.D. Anderson Hospital and Tumor Institute. Houston, Texas. Project: isolation of human placental RNA polymerase II.
- **1979-1982** Graduate student and predoctoral fellow in the laboratory of Dr. Grady F. Saunders at the M.D. Anderson Hospital and Tumor Institute. Houston, Texas. Project: Expression of the human placental lactogen genes.
- **1981** Genentech-UTHSC, Houston, intership and contributor to the world's record established by Drs.Elson and Chen determining greatest length of human nucleotide sequence (66,500 bp), cited in Genomics 4:479-497:1989 and "Research News" Science 2, December, 1988.
- **1983-1984** Postdoctoral fellow with Professeur Pierre Chambon, Laboratoire de Genetique Moleculaire des Eucaryotes du CNRS, Unite 184 de Biologie Moleculaire et de Genie Genetique del INSERM, Faculté de Medecine, Strasbourg, France.
- **1985** Consultant, Department of Biochemistry and Molecular Biology. The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, Texas.
- **Summer 1986** Visiting investigator and consultant in the Department of Biochemistry and Molecular Biology, M.D. Anderson Hospital and Tumor Institute, Houston, Texas.
- **Winter 1987** Visiting investigator in the Department of Gastrointestinal Oncology, M.D. Anderson Hospital and Tumor Institute, Houston, Texas.
- **Summer 1987** Visiting investigator in the Department of Pathology, School of Medicine of Temple University, Philadelphia, Pennsylvania.
- **Summer 1989** Postdoctoral visit to Dr. Daniel Bary´s biochemistry and molecular biology Center at the Centre National de Recherche Scientifique in Marseille, France.
- **Fall 1993** Visiting professor in the Molecular Biology Center of the Autonomous University of Madrid, Spain.
- **Fall 1994** Visiting professor in the Molecular Biology Center of the Autonomous University of Madrid, Spain.

PEER-REVIEWED ARTICLES PUBLISHED (695 citations via Dialog search of January 1994):

1. HARPER, M.E., BARRERA-SALDAÑA, H.A. and SAUNDERS, G.F. (1982). Chromosomal localization of the human placental lactogen-growth hormone gene cluster to 17q22-24., **Am. J. Hum. Genet.** 34:227-234
2. CALABRETTA, B., ROBBERSON, D.L., BARRERA-SALDAÑA, H.A., LAMBROU, T.P. and SAUNDERS, G.F. (1982). Genome instability in a region of human DNA enriched in Alu repeat sequences, **Nature**. 296:219-225
3. BARRERA-SALDAÑA, H.A., ROBBERSON, D.L. and SAUNDERS, G.F. (1982). Transcriptional products of the human placental lactogen gene, **J. Biol. Chem.** 257:12399-12404
4. BARRERA-SALDAÑA, H.A., SEEBURG, P.H. and SAUNDERS, G.F. (1983). Two structurally different genes produce the same secreted human placental lactogen hormone, **J. Biol. Chem** 258:3787-3793

5. BATY, D., BARRERA-SALDAÑA, H.A., EVERETT, R.D., VIGNERON, M. and CHAMBON, P. (1984). Mutational dissection of the 21 bp repeat region of the SV40 early promoter reveals that it contains overlapping elements of the early-early and late-early promoters, **Nucl. Acids. Res.** 12;915-932

6. VIGNERON, M., BARRERA-SALDAÑA, H.A., BATY, D., EVERETT, R.D. and CHAMBON, P. (1984). Effect of the 21-bp repeat upstream element on in vitro transcription from the early and late SV40 promoters **EMBO J.** 3:2373-2382.

7. RESENDEZ-PEREZ, D., BARRERA-SALDAÑA, H.A., MORALES-VALLARTA, M.R., RAMIREZ-BON, E., LEAL-GARZA, C.H., FERIA-VELAZCO, A. and SANCHEZ ANZALDO, F.J. (1984). Low-speed purification of human placental nuclei, **Placenta** 5:523-532.

8. BARRERA-SALDAÑA, H.A., TAKAHASHI, K., VIGNERON, M., WILDEMAN, W., DAVIDSON, I. and CHAMBON, P. (1985) All six GC-motifs of the SV40 early upstream element contribute to promoter activity in vivo and in vitro, **EMBO J.** 4:3839-3849.

9. GIDONI, D., KADONAGA, T., BARRERA-SALDAÑA, H.A., TAKAHASHI, K., CHAMBON, P. and TJIAN, R. (1985). Bidirectional SV40 transcription mediated by tandem Sp1 binding interactions, **Science,** 230:511-517.

10. CAB-BARRERA, E.L. and BARRERA-SALDAÑA H.A. (1988). Versatile plasmid vectors for use in studies of eukaryotic gene expression, **Gene** 70:411-413.

11. VARELA-ECHAVARRIA, A., MONTES DE OCA-LUNA, R. and BARRERA-SALDAÑA, H.A. (1988). Uricase protein sequences: conserved during vertebrate evolution but absent in humans, **FASEB J.** 2:3092-3096.

12. CAB-BARRERA, E.L. and BARRERA-SALDAÑA, H.A. (1989). A general method to Optimize the Amount of enzyme in restriction and DNA modification reactions using the Beta galactosidase blue-white plaques assay, **Biotechniques**, 7:132-136.

13. CHEN, E. Y., LIAO, Y. C., SMITH, D. H., BARRERA-SALDAñA, H.A., GELINAS, R. E. (1989). AND SEEBURG, P. H., The human growth hormone locus: nucleotide sequences, biology, and evolution. **Genomics**, 4:479-497.

14. RAMIREZ-SOLIS, R., RESENDEZ-PEREZ, D., ALVIDREZ-QUIHUI, L.E., RINCON-LIMAS, D., VARELA-MARTINEZ, R., MARTINEZ-RODRIGUEZ, H. AND BARRERA SALDAÑA, H.A. (1990). New vectors for the efficient expression of mammalian genes in cultured cells, **Gene,** 87:291-294.

15. RESENDEZ-PEREZ, D., AND BARRERA-SALDAÑA, H.A. (1990). Expression studies of transfected multigene families by homologous DNA mutagenesis, **Biotechniques,** 9:282-286.

16. RESENDEZ-PEREZ, D., RAMIREZ-SOLIS, R., VARELA-ECHAVARRIA, A. AND BARRERA-SALDAÑA, H.A. (1990). Coding potential of transfected human placental lactogen genes, **Nucleic Acids Research,** 18:4665-4670.

17. SILVA-CUDISH, J.B., GONZALEZ, N., VILLARREAL-GARZA, I. AND BARRERA-SALDAÑA, H.A. (1990). Informe de una familia con dos miembros que presentan la enfermedad de la orina de jarabe de arce. **Rev.Méd. del IMSS** 28:129-132.

18. VIADER-SALVADO, J.M., MARTINEZ-TORRES, A., MORENO-ROCHA, J.C., VILLALOBOS-ROMO, G., PICHARDO-MUÑOZ, L.V. AND BARRERA-SALDAÑA, H.A. (1991). Las microcomputadoras en la investigación científica. **Ciencia y Desarrollo** (CONACYT) Vol. XVII Núm. 100 pags. 128-138.

19. MARTINEZ-CAMPOS, A., HERNANDEZ, R.P., FORSBACH, G. AND BARRERA-SALDAÑA, H.A.(1991). The stimulatory effect of estradiol 17-ß on prolactin mRNA is inhibited by anti-calmodulin drugs. **Life Sciences,** 48:2475-2485.

20. GONZALEZ-GARAY, M.L., BARRERA-SALDAÑA, H.A., AVILES, L.B., ALVAREZ-SALAS, L.N. AND GARIGLIO, P.V. (1992). A comparative study of the incidence of human papillomavirus DNA sequences in cervical cancer from two mexican populations. **Rev. Inv. Clin.**44:491-499.

21. BARRERA-SALDAÑA, H.A., ROJAS-MARTINEZ, A., RIVERA-PEREZ, J.A., VAZQUEZ-ALEMAN, R.M. AND GONZALEZ-GARAY, M.L.(1992). Diagnóstico molecular de enfermedades hereditarias. Resease **Gaceta Médica de México**, 128:6 613-621.

22. ROJAS MARTINEZ, A., VAZQUEZ ALEMAN, R.M., CANTU GARZA, J.M. AND BARRERA SALDAÑA, H.A. (1992). Genética molecular de la fibrosis quística: el alelo -F50-8 en familias mexicanas. . **Bol.Med. Hosp.Infant.Méx.** 49:6 335-341.

23. BARRERA-SALDAÑA, H.A., ROJAS-MARTINEZ. A., RIVERA-PEREZ, J.A., VAZQUEZ-ALEMAN, R.M. AND GONZALEZ-GARAY, M.L. (1992). Diagnóstico molecular de enfermedades hereditarias. **Gaceta Médica de México**, 128:6 613-621.

24. RINCON-LIMAS, D.E., RESENDEZ-PEREZ, D., ORTIZ-LOPEZ, R., ALVIDREZ-QUIHUI, L.E., CASTRO-MUÑOZLEDO, F., KURI-HARCUCH, W., AND BARRERA-SALDAÑA, H.A.(1993). HGH isoform: cDNA expression, adipogenic activity and production in cell culture. **Biochimica et Biophysica Acta,** 1172: 49-54.

25. RIVERA-PEREZ, J.A., ROJAS-MARTINEZ, A., CHARLES-GARCIA, F. AND BARRERA-SALDAÑA, H.A. (1993). Análisis molecular de la hemofilia A en familias del Noreste de México. **Rev.Inv.Clin.** 45:23-28.

26. BARRERA-SALDAÑA, H.A., ORTIZ-LOPEZ, R., ROJAS-MARTINEZ, A. AND RESENDEZ-PEREZ, D. (1993). Reacción en cadena de la polimerasa: una nueva época dorada en la Biologíoa Molecular. **Ciencia y Desarrollo** (CONACYT) Vol.XVII, No.101 pags.68-80.

27. ASCACIO-MARTINEZ, J. AND BARRERA-SALDAÑA, H.A.(1994). A dog growth cDNA codes for a mature protein identical to pig growth hormone. **Gene**, 143:277-280.

28. ASCACIO-MARTINEZ, J. AND BARRERA-SALDAÑA, H.A.(1994). Sequence of a cDNA encoding horse growth hormone. **Gene** 143:299-300.

29. VILLARREAL-CASTELLANOS, E., VILLALOBOS-TORRES, M.C. AND BARRERA-SALDAÑA, H.A.(1994). Estudio genético molecular de fibrosis quística en una familia con cuatrillizos. **Bol.Med.Hosp.Infant.Méx.,** 51:5 337-340.

30 CASTRO-PERALTA, F. AND BARRERA-SALDAÑA, H.A. (1995). Cloning and nucleotide sequencing of cat growth hormone complementary DNA. **Gene** (in press).

31. SALINAS-MELENDEZ, J.A., TAMEZ-GONZALEZ, R., WELSH-LOZANO, O. AND BARRERA-SALDAÑA, H.A. (1995). Detection of borrelia burgdorferi DNA in human skin biopsies and dog synovial fluid by the polymerase chain reaction. **Rev.Lat.Microbiol.** (in press)

32. AMPUDIA-DE LA PUENTE, J.L., LAVALLE-GONZALEZ, F., CARDENAS-IBARRA, L., ESQUIVEL-ESCOBEDO, D., ROJAS-MARTINEZ, A., VILLARREAL-PEREZ, J. AND BARRERA-SALDAÑA, H.A. Analysis of HLA genotypes in insulin-dependent diabetes mellitus patients from northeastern Mexico. (**sent to Diabetes Care**).

REVIEWS

33. WALKER, W.H., FITZPATRICK, S.L., BARRERA SALDAÑA, H.A., RESENDEZ PEREZ, D. AND SAUNDERS, G.F. (1991). The human placental lactogen genes: Structure, Function, Evolution and Transcriptional Regulation. **Endocrine Reviews** 12:316-328.

BOOK CHAPTERS

34. ROBBERSON, D.L., CALABRETTA, B., BARRERA-SALDAÑA, H.A., SOMASUNDARAM, T., LAMBROU, T.P., STUBBLEFIELD, E., YEN, N., BROCK, D.L., PETERSON, C.A. and SAUNDERS, G.F. (1983). Genome rearrangements and extrachromosomal circular DNAs in human cells, In Perspectives on Genes and the Molecular Biology of Cancer G.F. Saunders and D.L. Robberson, Eds., Raven Press New York, pp. 51-80.
35. KIDD, V.J., BARRERA-SALDAÑA, H.A. and SAUNDERS, G.F. (1983). The human growth hormone and placental lactogen gene complex, In Perspectives on Genes and the Molecular Biology of Cancer, G.F. Saunders and D.L. Robberson, Eds., Raven Press New York, pp. 143-167.
36. SAUNDERS, G.F., CALABRETTA, B., ROBBERSON, D.L., BARRERA-SALDAÑA, H.A. and LAMBROU, T.P. (1984). DNA restriction fragment length polymorphisms in human leukemic leukocytes. In Research Perspectives in Cytogenetics, University Park Press, Baltimore, pp. 1.
37. WILDEMAN, A.G., ZENKE, M., SCHATZ, C., TAKAHASHI, K., BARRERA-SALDAÑA, H.A. GRUNSTROM, T., WINTZERITH, M., MATTHES, H., VIGNERON, M. and CHAMBON P. (1985). Interactions between the SV40 early promoter and cellular proteins. In Eukaryotic transcription: The role of cis-and trans-acting elements in initiation. Edited by Yakov Gluzman, Cold Spring Harbor, pp. 19-26.
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41. GONZALEZ-GARAY, M. L., GONZALEZ-GUERRERO, J.F. AND BARRERA-SALDAÑA, H.A. (1988). Detection of the Human Papillomavirus Genome in Cervical Cancer. In Cell Function and Disease L. Cañedo, L. Todd, J. Jaz and L. Packer, eds., Plenum Press, New York, pp. 333-341.
42. VARELA-ECHAVARRIA, A., CAÑEDO, L.E., AND BARRERA-SALDAÑA, H.A. (1988). Human Uricase Loss: An Evolutionary Gain Against Disease. In Cell function and disease L. Cañedo, L. Todd, J. Jaz and L. Packer, eds., Plenum Press, New York, pp. 219-223.
43. BARRERA SALDAÑA, H.A. (1991). Biología Molecular de un Gen Eucariote. In Temas Selectos en Biomedicina 1:43-67.
44. RAMIREZ-ANGULO, V., MARTINEZ-TORRES, A. AND BARRERA-SALDAÑA, H.A. (1992). Biología molecular y biotecnología de las hormonas del crecimiento animal. In Biotecnología Hoy. CONACYT pags. 111-132.
45. BARRERA-SALDAÑA, H.A.(1993). Producción de proteínas por Ingeniería Genética. In Enciclopedia Hematológica Ibero-Américana, Universidad de Salamanca. Spain.

BOOKS

46. BARRERA-SALDAÑA, H.A. (1992). Información Genética: estructura, función y manipulación. **CONACYT** Basic Science Colection. México.

LANGUAGES:

Spanish is my native language; English I understand and speak very well and can write with not much difficulty; French I can speak to be understood but have not had the necessity to write very much.

SCIENTIFIC SOCIETIES:

1985	Mexican Society for Biochemistry
1986	Mexican Society for Human Genetics
1991	Mexican Academy of Medicine
1991	Mexican Academy of Sciences
1991	Human Genome Organization (HUGO)
1992	American Society of Human Genetics
1992	Mexican Academy of Molecular Biology in Medicine

APPOINTMENTS AS REFEREE:

CONACYT grants; CONACYT Journal; GENE; BIOTECHNIQUES and REVISTA DE INVESTIGACION CLINICA.

#37

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.:	08/315,673	§	Docket No. D-5050-C3
Filing Date:	09/30/94	§	Examiner: A. Marschel
Applicant:	Caskey, et al.	§	Art Unit: 1807
Title:	Multiplex Genomic DNA Amplification for Deletion Detection	§	

Commissioner of Patents and Trademarks
Washington, D.C. 20231

DECLARATION OF FRANCESCO SALVATORE UNDER 37 C.F.R § 1.132

Dear Sir:

I, Francesco Salvatore, do hereby depose and say as follows:

1. I am a full professor of Human Biochemistry at the Dipartimento di Biochimica e Biotecnologie Mediche, Sezione di Biochimica e Biologia Molecolare in Napoli, Italy. I am skilled in the area of molecular biology and DNA amplification. My *curriculum vitae* is attached.

2. Using the protocol and materials provided by Dr. Caskey, multiplex amplification was performed successfully in my laboratory.

3. Prior to being provided with the methods and materials by Dr. Caskey, I had not performed multiplex reactions involving more than two sets of primers.

4. There was a need in the art for multiplex amplification using more than two sets of primers. Previously, screening for a plurality of DNA sequences required a large

number of separate assays for each sequence. The multiplex assay of Caskey et al. thus saves time and money.

5. My experience with Dr. Caskey's multiplex technique has led us to develop multiplex PCRs for other DMD exons and various genetic loci related to other diseases.

6. I hereby declare that all statements herein of my own knowledge are true, that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: January 23, 1995

Francesco Salvatore

CURRICULUM VITAE

FRANCESCO SALVATORE

Born 22 April 1934 in Naples, Italy

F. Salvatore was appointed Full Professor of Biochemistry at the Faculty of Medicine of the University of Naples in 1967. In 1989 he was appointed Full Professor of Human Biochemistry at the Medical School of the Università di Napoli Federico II.

From 1972 to 1982 he served as Director of the Institute of Biochemistry (Second School of Medicine and Surgery, University of Naples).

Visiting scientist and visiting professor for approximately 5 years, at:

- Argonne National Laboratory, Illinois, USA
- Institut National des Sciences et Techniques Nucléaires, Saclay, France
- Laboratoire de Biochimie Générale et Compareé, France
- European Molecular Biology Laboratory, Heidelberg, Germany.
- Department of Biological Science, University of Illinois, Chicago, Ill., USA
- Department of Biochemistry, University of California, Berkeley, USA
- Laboratory of Molecular Biology, Medical Research Council, Cambridge, UK.

After having conducted studies for more than twenty years in the fields of metabolic biochemistry and enzymology, to which he made original contributions to the nitrogen metabolism of amino acids, the ureogenetic cycle and the mechanism of detoxification of ammonia, in addition to methyltransferase and the metabolism of such sulphur compounds as adenosylmethionine, in the 1970s Prof. Salvatore began to investigate the biochemical and molecular biology aspects of nucleic acids.

In the early 1980s, in the Biochemistry Institute of the University of Naples, he developed studies on the molecular biology of transfer RNAs, and studies on the structure and expression of human protein genes including those of the aldolase system and of glyceraldehyde-3-phosphate-dehydrogenase. These studies led to a series of important results through the elucidation of complete gene sequences as well as to the sequences linked to the promotor region for gene expression. Also in the 1980s he began studies in Clinical Biochemistry and his research groups soon became internationally recognized for their work on the differential diagnosis in man of clinically confounding disorders such as neoplasias by laboratory indicators.

In more recent years, Prof. Salvatore focused on research on the molecular biology of gene expression, and in the Dipartimento di Biochimica e Biotecnologie Mediche, he has combined, thanks also to the constitution of the Centre for Genetic Engineering (CEINGE) of which he is President, the aims of clinical biochemistry with those of molecular biology through the study of molecular alterations at gene level, thus contributing to the molecular diagnosis of hereditary and acquired genetic disorders, and contributing to the foundation in his area of "Clinical Molecular Biology".

Professor Salvatore has presented his findings by invitation at numerous national and international meetings, and has published about 200 in extenso papers, many of which in international peer-reviewed scientific journals, e.g., Bull. Soc. Chim. Biol. (Paris)., Nature, Biochimica Biophysica Acta, Clinica Chimica Acta, Biochemical Pharmacology, Life Sciences, Archives of Biochemistry and Biophysics, Experimental Cell Research, Enzymology, Analytical Biochemistry, International Journal of Biochemistry, Nucleic Acid Research, Molecular and Cellular Biochemistry, Cancer Research, Cancer Detection and Prevention, Journal of Chromatography, European Journal of Biochemistry, Clinical Chemistry, Clinical Biochemistry, Journal of Tumor

Marker Oncology, Human Genetics, Biochemical Biophysical Research Communication, FEBS Letters, Electrophoresis, European Journal of Clinical Chemistry and Clinical Biochemistry, Biochemical Genetics, and EMBO Journal.

Professor Salvatore is Co-Editor-in-Chief and Managing Co-ordinator of the Bulletin of Molecular Biology and Medicine and has edited or co-edited the following volumes:
- Macromolecules in the Functioning Cell, Plenum Press, London/New York (1979).
- Biochemical and Pharmacological Roles of Adenosylmethionine and the Central Nervous System, Pergamon Press, Amsterdam (1979).
- Biochemistry of Adenosylmethionine, Columbia University Press, New York (1982).
- Reference Values and Predictive Values in Laboratory Medicine, Bulletin of Molecular Biology and Medicine (vol. 10, 1983).
- Novel Aspects in Clinical Biochemistry, Bulletin of Molecular Biology and Medicine (Vol.10, 1983).
- Human Tumor Markers, Walter de Gruyter, Berlin/New York (1987).
- Clinical Biochemistry in Hepatobiliary Diseases, Progress in Clinical Biochemistry & Medicine.vol 8. Springer-Verlag, Berlin/Heidelberg (1989).

Professor Salvatore is a member of the Editorial Board of Clinica Chimica Acta and of the International Journal of Human Tumor Markers.

Professor Salvatore has been or is a member of the following Committees:

- Biotechnology Committee of the Italian Ministry of Health.
- National Committee for Scientific Research and Technology (Biology) of the Italian Ministry of Education and Scientific Research (MURST).
- MURST Committee for the new degree course in Biotechnology.
- Italian Ministry of Health Committee for Laboratory Medicine (1991-92).
- MURST Expert Committee to evaluate projects of the National Programme of Research and Formation on Neurobiology-Technology Systems of Signal Transduction.
- Italian Interministry Coordination Committee. Implementation of EC directives 219/90 and 220/90.
- Italian Ministry of Health Working Group on Laboratory Medicine (1993).
- Technical-Consulting Committee of the Italian Ministry of Health for the law (ex art. 16 of DPCM of 10.2.84).
- Member of the Regional Committee and Coordinator of projects related to "Congenital Disorders" for Health Research of the Regione Campania.
- Consultant for the III Section of the Consiglio Superiore di Sanità for Laboratory Medicine.

From 1976 to 1980 he was Secretary of the Commission for International Relationships of the Italian National Research Council (CNR).

He was a member of the Scientific Committee of the Progetto Finalizzato CNR Oncologia for five years (1985-1989), and is a member of the Project Committee of the CNR Progetto Applicazioni Cliniche alla Ricerca Oncologica for a further five years (1990-1995). He has been appointed by the CNR as a member of a committee to assess the feasibility of a 5-year national research project on human genoma and recombinant DNA.

Professor Salvatore is President and General Coordinator of scientific research of CEINGE-Biotecnologie Avanzate (Naples), which is a consortium founded in 1983 for applied research in the field of biotechnology, and in particular, recombinant DNA.

Professor Salvatore is member and President of the Technical Promoting Committee of the new Faculty of Mathematical, Physics and Natural Sciences of the University of Molise (Isernia).

Societies

Società Italiana di Biochimica (from 1970 to 1976, Member of the Executive Committee and Treasurer from 1974 to 1976); Società Italiana di Biochimica Clinica (SIBioC) from 1972 (President from 1985 to 1989; Member of the Executive Committee from 1983); American Chemical Society; American Society for Microbiology; American Association of Clinical Chemistry; The Biochemical Society; The Royal Society of Medicine; Academy of Clinical Biochemistry; International Academy of Tumor Markers; The Society for the Study of Inborn Errors of Metabolism.

#37

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Ser. No.:	08/315,673	§	Docket No. D-5050-C3
Filing Date:	09/30/94	§	Examiner: A. Marschel
Applicant:	Caskey, et al.	§	Art Unit: 1807
Title:	Multiplex Genomic DNA Amplification for Deletion Detection	§	

Commissioner of Patents and Trademarks
Washington, D.C. 20231

DECLARATION OF LENNIE PINEDA DE DEL VILLAR UNDER 37 C.F.R § 1.132

Dear Sir:

I, Lennie Pineda de Del Villar, do hereby depose and say as follows:

1. I am the Director of the Unidad De Genetica Medica, Facultad de Medicina, at the Universidad Del Zulia, Apartado 15066, Maracaibo, Venezuela. I am skilled in some aspects and procedures of molecular biology and DNA amplification which are very interesting to our laboratory. I have obtained my knowledge taking short courses (please see my *curriculum vitae*, attached).

2. Using the protocol and materials provided by Dr. Caskey, multiplex amplification was performed successfully in my laboratory.

3. Prior to being provided with the methods and materials by Dr. Caskey, I had not performed multiplex reactions involving more than two sets of primers.

4. There was a need in the art for multiplex amplification using more than two sets of primers. Previously, screening for a plurality of DNA sequences required a large

number of separate assays for each sequence. The multiplex assay of Caskey et al. thus saves time and money.

5. My experience with Dr. Caskey's multiplex technique has facilitated the standardization of other procedures regarding multiplex PCR of the CTFR gene.

6. I hereby declare that all statements herein of my own knowledge are true, that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: January 20th 1995

Lennie Pineda

Lennie Pineda de Del Villar

Dirección: Hospital Universitario Aptdo. 1154 - Teléfonos: 525044 - 5250[redacted] - 519496 - Maracaibo - Venezuela

CURRICULUM VITAE (Short CV.)

A) DATOS PERSONALES
NOMBRE: Lennie Pineda de Del Villar.
CEDULA DE IDENTIDAD: 3.277.187.
LUGAR DE NACIMIENTO: Maracaibo, Estado Zulia.
DIRECCION: Urb. El Portal. Calle 50. #12-118. Maracaibo, Edo Zulia. Teléfono Hab: 61-421543/Ofi: 61-421543.

B) ESTUDIOS UNIVERSITARIOS

1. UNIVERSITARIOS
Licenciada en Enfermeria. Escuela de Enfermeria. Facultad de Medicina. Universidad del Zulia. 1966-1970 (4 años).

2. CURSOS DE POSTGRADO.

2.1 Curso Intensivo Nacional de Demografia. Facultad de Ciencias Economicas y Sociales. Universidad del Zulia. 1973 (4 meses)

2.2 Curso Medio de Salud Publica. Escuela de Salud Publica. Universidad Central de Venezuela. 1974 (4 meses)

2.3 MAESTRIA.
Curso de Magister Scientiarum en Biologia. Mención Genética Humana. Intituto Venezolano de Investigaciones Cientificas 1975-1977 (2 años y 7 meses). Tesis: Hibridación en un aislado genético de origen alemán.

3. OTROS CURSOS.

3.1 Entrenamiento en el laboratorio de Genética del Instituto de Zoologia y Genética de la Universidad de Ferrara. Italia: 1 Diciembre 1983 - 10 Febrero 1984.

3.2 Curso sobre Técnicas Básicas de ADN Recombinante. Laboratorio de Biología Molecular. Instituto de Medicina Experimental. Universidad Central de Venezuela: 16 al 27 de Septiembre de 1991.

3.3 Curso en Gerencia en Ciencia y Tecnología. Auspiciado por CONICIT-FUNDACITE-LUZ: 27 de Junio al 13 de Julio de 1991. (40 horas).

3.4 IV Curso de Ingeniería Genética y Biotecnología. Asociación Venezolana de Bioquímica. Maracaibo: 25 al 29 de Noviembre de 1991.

3.5 Curso teórico-practico sobre Reacción en Cadena de la Polimerasa y sus aplicaciones al estudio del Genoma Humano. Instituto Mexicano del Seguro Social. Guadalajara: 24 al 28 de Febrero de 1992.

3.6 Curso teorico-práctico sobre Reacción en Cadena de la Polimerasa y su aplicación al estudio de enfermedades hereditarias. Unidad de Genética Médica. LUZ: 18 al 22 de Enero de 1993.

3.7 Curso teórico práctico sobre Hibridación Fluorescente in Situ. Universidad Autonoma de Nuevo León. Monterrey. Mexico: 11 al 15 de Octubre de 1993.

3.8 Curso teórico práctico sobre Diagnóstico de enfermedades hereditarias por la Reacción en Cadena de la Polimerasa. Universidad Autónoma de Nuevo León. Monterrey. Mexico: 18 al 22 de Octubre de 1993.

C) PREMIOS

Premio ANDRES BELLO en el area de Ciencias Exactas y Naturales, año 1986. Otorgado por la Universidad del Zulia por el trabajo de investigación: Anomia Falciforme en Isla de Toas. Zulia.

D) CARGOS DESEMPEÑADOS

1. Directora del Programa de Maestria en Genética: Mención Genética Médica de la Unidad de Genética Médica. Facultad de Medicina. Universidad del Zulia. Septiembre 1991 hasta el presente.
2. Coordinadora Titular de la Unidad de Genética Médica de la Facultad de Medicina de la Universidad del Zulia desde el 4 febrero de 1991 hasta el presente.
3. Profesor de de la Unidad de Genética Médica de la Universidad del Zulia, desde el 1 de noviembre de 1979, actualmente con la categoria de Titular.
4. Contratación por el Instituto Venezolano de Investigaciónes Cientificas. Caracas, desde Marzo de 1979 al 15 de Septiembre del mismo año.
5. Contratación por CONICIT para el laboratorio de Genetica Humana del Instituto de Investigaciones Cientificas. Caracas desde Marzo de 1978 a Febrero de 1979.
6. Profesor Ordinario de la Unidad de Genética de la Universidad del Zulia desde el primero de Mayo de 1972 al 22 de Marzo de 1977.
7. Becario docente y de investigación de la Cátedra de Biologia II (Genética Médica). Universidad del Zulia. Cargo ganado por concurso y desempeñado desde el 1 de Mayo de 1972 al 30 de Abril de 1974.

E) DISTINCIONES

Ingreso al Programa de Promoción al Investigador del CONICIT desde Julio 1991 a Julio 1993. Nivel I

F) PUBLICACIONES EN REVISTAS CIENTIFICAS.

1. Pineda B., L. Niveles de prevención y enfermedades hereditarias. Boletin de la Unidad de Genética Médica del Estado Zulia: 1 (2): 13-15, 1974.
2. Pinto Cisternas J., Castelli M.C., Pineda B, L. La consaguinidad en la Parroquia de los Teques, Venezuela. Desde 1790-1869. Acta Cient Venez 32:262-268, 1981.
3. Pineda de Del Villar, L. La hemoglobinopatia S en Isla de Toas. Rev de la Fac de Med. Maracaibo 17(1-4):169-210, 1985.
4. Pinto Cisternas J., Pineda L., Barrai I. Estimation of inbreeding by isonimy in Iberoamerican populations: An extension of the method of Crow and Mange. Am J Hum Genet 37: 373-385. 1985.
5. Pineda L., Pinto Cisternas J., Arias S. Consanguinity evolution in Colonia Tovar. A venezuelan isolated settlemet of german origin. (1843-1977). J Hum Evol 14: 587-596, 1985.
6. Pinto Cisternas J., Castelli M.C., Pineda L. Use of surnames in the study of population structure. Hum Biol 57(3): 353-363, 1985.
7. Pineda de Del Villar L., Borjas L. La hemoglobinopatia S

24-28, 1987.

9. Pineda L., Villalobos H., Barrai I. The origin of the HbS gene in the Island Toas, Venezuela. Inter J Anthropol 3(1): 1-8, 1988.
10. Callejas D., Pineda L. Identificación Fenotipica de la G6-PD en la población estudiantil de Isla de Toas. Rev Latinoamericana de Genética 2(1): 35-41, 1988.
11. Villalobos de R.A., Angarita L., Herrera de M.R., Pineda L. Influencia de la edad y el sexo en los componentes tres y cuatro (C'3 y C'4) del complemento serico en población normal del Distrito Maracaibo. Kasmera 17(1-4): 55-66, 1989.
12. Pineda de Del Villar, Chacón I., Villalobos M. Evidencia genealogica de control genético en el Sindrome de Down. Acta Científica Venezolana 42(3):134-137.1991.
13. Pineda de Del V.,L.,Navarro G.,Del Villar A. Defectos del Tubo Neural en el Hospital Pedro García Clara. Estado Zulia: 1982-1988. Invest Clín 34(1):41-52, 1993.
14. Rojas de A. A., Roldán de P.,Pineda L. Importancia de las alteraciones cromosómicas en Leucemia Mieloide Crónica. Invest Clín 34(2), 1993.
15. Pineda de Del V.,L, Martinez M.C., Delgado W. Epidemiología de malformaciones congénitas en el Hospital Pedro García Clara. Ciudad Ojeda. Estado Zulia. Invest Clín 35(1),1994
16. Delgado W.,Pineda de Del V.,L.,Borjas L.,Martinez C.,Barrera S.,H. Diagnóstico molecular de Distrofia Muscular Duchenne/Becker mediante la Reacción en Cadena de la Polimerasa. Aceptado en la revista Invest Clín.

#32

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Ser. No.:	08/315,673	§	Docket No. D-5050-C3
Filing Date:	09/30/94	§	Examiner: A. Marschel
Applicant:	Caskey, et al.	§	Art Unit: 1807
Title:	Multiplex Genomic DNA Amplification for Deletion Detection	§	

Commissioner of Patents and Trademarks
Washington, D.C. 20231

DECLARATION OF GERT-JAN B. VAN OMMEN UNDER 37 C.F.R § 1.132

Dear Sir:

I, Gert-Jan B. van Ommen, do hereby depose and say as follows:

1. I am the Head of the Department of Human Genetics at the Medical Genetics Center South-West Netherlands, at Wassenaarsoweg 72, 2300 RA Leiden, The Netherlands. I am skilled in the area of molecular biology and DNA amplification. My *curriculum vitae* is attached.

2. Using the protocol and materials provided by Dr. Caskey on January 26, 1989, multiplex amplification was performed successfully in my laboratory, and has assisted greatly the diagnostic abilities of my laboratory.

3. Prior to being provided with the methods and materials by Dr. Caskey, I had not performed multiplex reactions involving more than two sets of primers.

4. There was a need in the art for multiplex amplification using more than two sets of primers. Previously, screening for a plurality of DNA sequences required a large

number of separate assays for each sequence. The multiplex assay of Caskey et al. thus saves time and money.

5. My experience with Dr. Caskey's multiplex technique has led to other operational multiplex systems.

6. I hereby declare that all statements herein of my own knowledge are true, that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 30 jan 1995.

Gert-Jan B. van Ommen

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231. on Oct. 3, 1995

LINDA A. BOURG
Name of applicant, assignee, or Registered Representative

Linda A. Bourg 10/3/95
Signature Date

0476093

Curriculum Vitae
Professor dr. Gert-Jan B. van Ommen, PhD.
Head of Department, Department of Human Genetics, University Leiden, The Netherlands

Born	09-28-1947 in Amsterdam, The Netherlands.
1959-1964	High School, Amsterdam.
1969	BSc Chemistry, Physics and Biology, University Amsterdam.
1974	MSci Cum Laude Biochemistry, University Amsterdam.
1980	PhD thesis 'RNA Synthesis in Yeast Mitochondria', promotor Prof.dr. P. Borst, University of Amsterdam.
1979-1983	Post-doctoral fellow with the Pediatric Department of the Academic Hospital of the University of Amsterdam. Research Project: Congenital hypothyroidism, due to disorders in the human and goat (model system) genes for thyroglobulin, the protein precursor for the thyroid hormones.
1983	Post-doctoral fellow in the Department of Human Genetics, University of Leiden, The Netherlands, headed by Prof.dr. P.L. Pearson. Research project: DNA analysis into aetiology and diagnosis of Duchenne and Becker muscular dystrophy.
1985	Associate co-ordinatorship of research into aetiology and diagnosis of Huntington's disease.
1987	Tenure position as of January 1 as scientific officer with the Clinical Genetic Center of the University of Leiden, as head of the DNA research section in the Department of Human Genetics, including specifically DMD/BMD, HD and Polycystic Kidney Disease, and long-range genome mapping of chromosomes Xp and 4p.
1989	Head of the laboratory and scientific coordinator of the Department of Human Genetics in the Medical Genetics Center of Southwest Netherlands.
1990	Vice-chairman of the Department of Human Genetics.
1991	Head of the Department of Human Genetics.

Awards

1993	The Gaetano Conte Prize 1993 for basic research on muscular diseases.

Editorships

- Molecular and Cellular Probes, Academic Press (N. Hales, D. Yolken)
- Technique, Saunders (P. Little)
- NeuroMuscular Disorders, Pergamon Press (V. Dubowitz)
- Human Molecular Genetics, Oxford University Press (KE Davies, HR Willard)
- Journal of Medical Genetics, BMJ Publishing Group (P. Harper)
- Genome Data Base, Baltimore (PL Pearson): Chromosome 4 editor
- European Journal of Human Genetics (G. Romeo)
- Acta Cardiomyologica (G. Nigro)

Memberships, international

- Senior editor of HUGO Chromosome 4 Committee
- HUGO Council member
- HUGO Europe trustee (1994: vice-president)
- HUGO/HGMW Human Genome Mapping Workshop Executive Committee
- Chairman of HUGO YAC library committee
- HUGO Travel Award committee
- The Wellcome Trust, Genetics Interest Group
- Faculty member of European School of Medical Genetics, Sestri Levante, Italy
- Scientific advisory board EAMDA (Eur. Assoc. Musc. Dyst. Assoc.)
- Distiguished contact person for the Mediterranean Society of Myology

Memberships, national

- Board Medical-Genetic Center South West Nederlands
- Scientific Advisory Council Medical-Genetic Center South West Nederlands
- Advisory Board Stichting Klinische Genetica Leiden (Foundation Clinical Genetics Leiden)
- Faculty Council Medical Faculty Leiden
- Education Committee grades 2-3-4 Medical Faculty Leiden
- Chairman of the Dutch Society for Human Genetics
- Board Stichting Onderzoek Neuromusculaire Ziekten (Research Foundation Neuromuscular Disorders)
- Scientific Advisory Board of the Dutch Society for Huntington's Disease
- Committee Genetic Screening of the Dutch Health Council
- NWO Committee Celbiology and Genetics
- NWO Committee Chronic Diseases, subcommittee Strategic Research

Organisation congresses

- Program Advisory Board Human Genome Meeting 1993
- Scientific Program Committee Human Genome Meeting 1996
- Organisatory Board 2nd HUGO Conference 'Human Genome Diversity', Alghero, Italy 1992
- Steering group NWO/EEC/NIH Meeting 'Decade of the Brain', The Hague, The Netherlands 1992
- Organiser of ENMC Workshop on neonatal screening for DMD/BMD, Soest, The Netherlands 1992
- Organiser of 2nd International Chromosome 4 mapping workshop, Leiden, The Netherlands 1992
- Organiser of MGC/BODL Advanced Course in Molecular Genetics 'Genome Research in Cancer and Genetic Diseases', Leiden, The Netherlands 1992
- Organiser of 3rd International Chromosome 4 mapping workshop, Stanford, USA 1993
- Organiser of MGC/BODL Advanced Course in Molecular Genetics 'Genome Research in Cancer and Genetic Diseases', Leiden, The Netherlands 1993

#37

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No.:	08/315,673	§	Docket No.: D-5050-C3
Filing Date:	30 September 1994	§	Examiner: A. Marschel
Applicant:	Caskey, et al.	§	Art Unit: 1807
Title:	Multiplex Genomic DNA Amplification for Deletion Detection	§	

Box Non-Fee Amendment
Assistant Commissioner of Patents
Washington, D.C. 20231

DECLARATION OF JEFFREY S. CHAMBERLAIN UNDER 37 C.F.R. §1.132

Dear Sir:

I, Jeffrey S. Chamberlain, Ph.D., do hereby depose and say as follows:

1. I am an Associate Professor in the Department of Human Genetics at the University of Michigan Medical School.

2. I am a co-inventor of the invention that is the subject of U.S. Serial No. 08/315,673, filed September 30, 1994, which claims a priority date of October 12, 1988. The other inventors of U.S. Serial No. 08/315,673 are Charles Thomas Caskey, Richard A. Gibbs, Joel E. Ranier Chamberlain and Phi Nga Nguyen. I have read U.S. Serial No. 08/315,673, and I am aware of its contents.

3. Furthermore, I am a co-author of the Chamberlain, et al., *Am. J. of Human Genetics*, Vol. 43, Abst. No. 711 (1988) publication. The other authors of the *Am. J. of*

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to Assistant Commissioner of Patents Washington, D.C. 20231 on Oct. 3, 1995
Linda A. Bourg
Linda A. Bourg 10/3/95
Signature Date

Human Genetics abstract are Richard Gibbs, Joel Ranier, Phi Nga Nguyen, Nancy J. Farwell and C. Thomas Caskey.

4. The Chamberlain et al. abstract in *Am. J. of Human Genetics* has been cited by the Examiner as a basis for rejecting claims 1-8 and 18-24 under 35 U.S.C. § 102(a) in the Office Action of April 4, 1995. The Examiner claims the invention was known or used by others in this country due to the difference in inventorship of the present invention and authorship of the abstract. Specifically, Nancy Farwell is listed as an author of the abstract but not as an inventor of U.S. Ser. No. 08/315,673. Nancy Farwell worked in the capacity of a research technician, and assisted the inventors with their experiments. Ms. Farwell was not involved in planning experiments or interpreting data. She was not involved in establishing multiplex PCR; she helped only in screening a genomic library to isolate gene fragments which were used ultimately by the inventors to establish the invention. Ms. Farwell was supervised by me and worked at my direction; thus, she acted only in a technical capacity and is not an inventor of the subject invention. Thus, the *Am. J. of Human Genetics* abstract is not a proper reference under 35 U.S.C. § 102(a) as the invention was not known or used by others in this country.

5. As the person signing this declaration, I hereby declare that all statements herein of my own knowledge are true, that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the applications or any patent issued therein.

Date: Sept. 27, 1995

Dr. Jeffrey S. Chamberlain, inventor

Attach to #37

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Ser. No:	08/315,973	§	DOCKET D-5050-C3
Filing Date:	09/30/94	§	EXAMINER: A. MARSCHEL
Applicant:	Caskey, et al.	§	
Title:	Multiplex Genomic DNA Amplification for Deletion Detection	§	Art Unit: 1807

Commissioner of Patents and Trademarks
Washington D. C. 20231

DECLARATION OF JULIAN GORDON UNDER 37 C.F.R. §1.132

Dear Sir:

I, Dr. Julian Gordon, do hereby state as follows:

1. I am Senior Research Fellow of the Volweiler Society of Abbott Laboratories, Abbott Park, Illinois. I am skilled in the area of molecular biology and DNA amplification. My curriculum vitae is attached.

2. I have carefully reviewed a copy of the DMD Multiplex Protocol (hereafter "Protocol") which is attached hereto as Exhibit 1. I have also carefully reviewed claims 1, 20, 21 and 22 of the above-referenced application, in the current form in which they are being prosecuted.

3. I have made a comparison of the Protocol with each of the process recitations in the above-referenced claims to determine whether all of the steps of these claims are carried out in the Protocol.

4. My technical conclusion, based on the comparison referred to in paragraph 3 above, is that the Protocol corresponds to the methods described in the above-referenced claims and that carrying out the Protocol will result in performance of each of the steps recited in claims 1, 20, 21 and 22. A detailed summary of my

conclusions in the form of a side-by-side comparison is presented in Exhibit 2 attached hereto.

5. I have carefully read the specification of the above-referenced application. In my technical opinion, it is clear from the specification, taken as a whole, and particularly from Examples 1 and 2, that the use of primers having similar melt temperatures is an important feature of the present invention, and that a person of ordinary skill in this field would clearly recognize this from reading the specification. In particular, I view the passage on page 16, lines 15 to 34, to be particularly relevant when read in conjunction with Examples 1 and 2 in their entirety, insofar as it clearly, if implicitly, teaches that the balancing of primer Tm's is an important feature of the present invention.

6. I hereby declare that all statements herein of my own knowledge are true, that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

Date: September 21, 1995

Julian L Gordon

Julian Gordon, Ph.D

I, hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231. on Oct. 3 1995

LINDA A. BOURG

Name of applicant, assignee, or Registered Representative

Linda A. Bourg 10/3/95

Signature Date

EXHIBIT 1

Dear Colleagues,

Enclosed are 50 of the Reaction mixes for multiplex amplification of the DMD gene. Each tube contains 45µl of reagent, allowing for addition of template DNA to a final volume of 50µl. I am also enclosing the protocol that we are using for amplification, a map of the gene indicating where each of the amplified regions are located and which exons are included in each fragment, a photograph of an agarose gel showing what successful reactions look like, two aliquots of control DNAs, and a few helpful tips. If you have any questions whatsoever or encounter any difficulties please immediately call either myself or Ms. Joel Ranier and we will assist you in any way we can. Each batch of reagents has been individually tested against a positive and a negative control. We suggest that you initially test the kits against one or both of the control DNAs we have sent to ensure that your enzyme, thermocycler, and gels are working adequately, and that there has not been any problem with shipping the mixes. The mixes should be stored at -70 degrees and should not be freeze-thawed. Looking forward to hearing of your experiences.

Jeffrey S. Chamberlain, Ph.D.
2/2/89

Multiplex DNA Amplification

Jeffrey S. Chamberlain
Joel E. Ranier
Richard A Gibbs
1/23/89

1. Prepare template DNA from lymphoblasts, amniotic fluid cells, or CVS dissected of decidual tissue using standard protocols.

2. Add to a 0.5 ml microfuge tube: H_2O to 45 µl final volume, 10 µl 5X *Taq* polymerase buffer (83 mM $(NH_4)_2SO_4$; 335 mM Tris-HCl, pH 8.8; 33.5 mM $MgCl_2$; 50 mM β-mercaptoethanol; 850 µg/ml bovine serum albumin; and 34 µM EDTA), 3 µl 25 mM each dNTP (Pharmacia), 25 pmols of each oligonucleotide primer, and then 5 µl of DMSO. The reaction mixes are stable at -70 °C for up to 3 months. (This is the premade mix).

3. Mix gently; add 250 ng template DNA. (Dilute DNA to a final concentration between 50 and 250ng/µl so that the DNA may be added to the microfuge tube in a volume of 5µl or less.) Add H_2O to a final volume of 50µl.

4. Add 5 units *Taq* polymerase (Perkin Elmer/Cetus), mix gently.

5. Add 25 µl paraffin oil, centrifuge 5 seconds.

6. Place sample in an automatic thermocycler. Cycle as follows: A. 94 °C X 6 min-(once); B. 94 °C X 30sec; 56 °C X 30 sec; 65 °C X 4 min (repeat 23 times) C. 65 °C X 7 min-(once) D. 4 °C until analysis (up to two months)

7. Electrophorese 15 µl of the reaction products on a 1.4% agarose or a 3% NuSieve® GTG agarose gel(FMC Bioproducts) (containing 0.5 µg/ml ethidium bromide) for two hours at 3.7 V/cm in 90 mM Tris-base, 90 mM boric acid, 1 mM EDTA (TBE).

8. Photograph the gel or otherwise record the results.

Notes

A) For use of the kits, simply thaw a reaction mix, add 250ng DNA and H_2O to a final volume of 50µl. Proceed to step 4. The amount of DNA is fairly important to get good results. If you've added too little and get faint bands you can put the tube back on the thermocycler for a few additional cycles (up to a week later even), no additional enzyme is required. Too much DNA throws off the ratio of the bands, and greatly increases the danger of false positive amplification from maternal or contaminating DNA.

B) Getting good resolution and a 'pretty' agarose gel can sometimes be tricky. Although this may be obvious to everyone, these are some of the things we have done to get the best looking gels. For quicker, cleaner resolution of the amplified fragments pouring a 3% NuSieve® GTG gel is recommended. When dissolving the agarose it is important to stir the mixture of the agarose and running buffer to eliminate any trapped air at the bottom of the flask. If the agarose and buffer are not mixed well some of the agarose will not go into solution. To dissolve the NuSieve® GTG agarose in a microwave oven, heat for 2 minutes on MEDIUM (70% full power). Swirl gently and bring the solution to a boil on HIGH for 30 seconds. When dissolving on a hot plate heat slowly until the agarose dissolves then bring to a boil. Cool the solution to 55°-65°C before pouring. In loading the gel be sure either to remove the mineral oil layering the sample before taking an aliquot or wipe, with a Kimwipe, the pipette tip after pipetting the sample to remove oil that has adhered to the outside of the tip. The oil will interfere with the migration of the DNA through the gel.

C) The control DNAs are: a) normal control female DNA, b) a partial deletion, Sample C in the photograph we have enclosed.

D) It is critical to avoid contamination of the reactions with exogenous or maternal DNA. You must physically separate the preparation and analysis stages of the reactions. Thus amplified reactions are opened and aliquots removed for analysis at a separate location to where the reactions are initiated. In addition, separate pipettors are used with the amplified reactions than are used to mix together the initial ingredients. These precautions are critical to prevent minute quantities of prior reaction products from serving as efficient template for future reactions.

Sample contamination must also be kept to a minimum. Equipment that has

been in contact with either prior reaction products or cloned DNA complementary to any of the regions targeted for amplification must not be used to prepare template DNA. We prepare all DNA samples for PCR analysis on an Applied Biosystems model 340A DNA extractor. Preparation of several hundred samples on this machine has not yet led to any detectable contamination from either exogenous or prior sample sources. Standard methods will work just fine though, as long as the above stated care is taken. Maternal DNA contamination of amniotic fluid cells or chorionic villus specimens (CVS) is a problem less easily controlled by laboratory personnel. CVS routinely should be dissected of maternal decidual tissue prior to extraction of DNA. Beyond this care must be taken to ensure that amplification is kept to the absolute minimum required to make a diagnosis. By performing reconstitution experiments with normal and partially deleted DNA samples we have observed that levels of maternal DNA at up to 5% of the total will not lead to false positive amplification as long as the reactions do not approach saturation [JSC et al, *Nucleic Acids Res.* 16, 11141-11156 (1988)]. Under the specified conditions we have not yet observed false-positive amplification during analysis of approximately 25 amniotic fluid cell or CVS DNA samples.

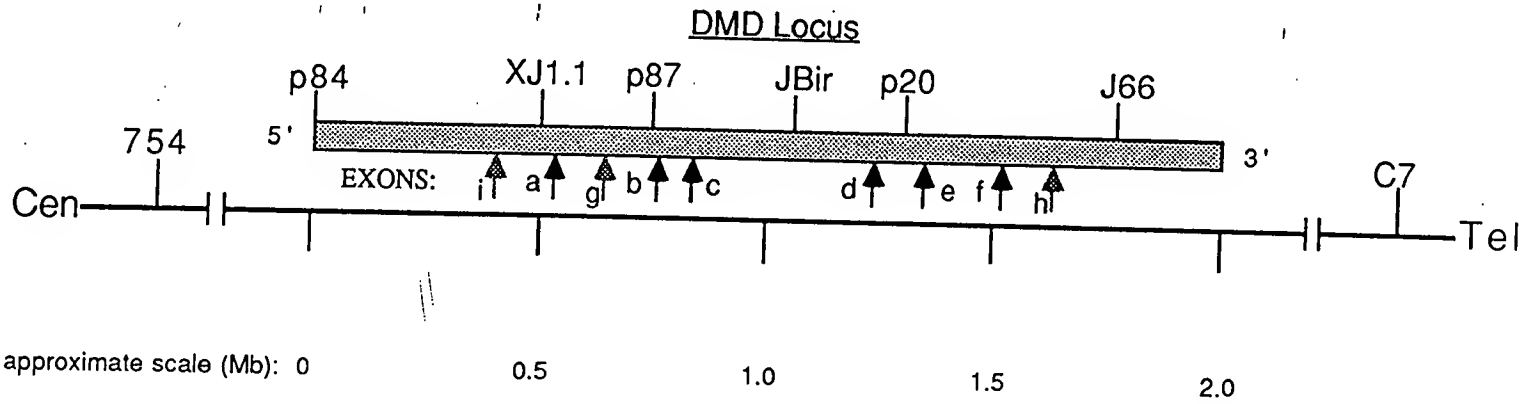
To eliminate or preclude the possibility of contaminated pipettors they may be effectively cleaned of DNA as follows. Soak the barrel of your pipetman in a beaker with 0.25N HCl for 30 min., repeat with 0.5N NaOH for 30 min, rinse well with distilled water, dry.

E) For the purpose of the collaboration every sample that you wish included in the manuscript must have corroborative Southern data. For PCR detected deletions, the deletion must be confirmed via Southern analysis although it may not be necessary to use the entire cDNA, only a region within the deletion. Unfortunately for non-deleted samples the entire cDNA will have to be used to arrive at accurate numbers on the percentage of all deletions detected via PCR. HindIII digested DNA must be used for this study so that everyone's data is compatible. The BglII hybridization pattern is not yet complete, so direct correspondence between Southern and PCR results would not be possible.

F) The revised order of the hybridizing HindIII fragments at the 3' end of the DMD gene shown on the reporting sheet that you were sent last week was determined by myself and is now published in *J. Clin Inv.* 83:95-99 (1989).

MULTIPLEX DNA AMPLIFICATION AT THE DMD GENE

-primer sets flanking nine exons



MW A B C D E F —

Fragments
(top to bottom)

e
f
c
b
h
a
g
d
i

EXONS

i) exon 4
a) exon 8
g) exon 12
b) exon 17
c) exon 19
d) 4.1kb HindIII (probe 7)
e) 0.5kb HindIII (probe 7)
f) 1.2/3.8kb HindIII (probe 8)
h) 3.1kb HindIII (probe 8)

Table 1. Summary of DMD gene multiplex amplification primer sets.

Exon and Size[1]	Primer Sequence[2]	Amplified[3]
A. Exon8 182bp; (probe 1b)	F- GTCCTTTACACACTTTACCTGTTGAG R- GGCCTCATTCTCATGTTCTAATTAG	360 bp
B. Exon17 178bp; (probe 3)	F- GACTTTCGATGTTGAGATTACTTTCCC R- AAGCTTGAGATGCTCTCACCTTTTCC	416 bp
C. Exon 19 88bp; (probe 3)	F- TTCTACCACATCCCATTTTCTTCCA R- GATGGCAAAAGTGTTGAGAAAAAGTC	459 bp
D. 4.1Kb HindIII 148bp; (probe 7)	F- CTTGATCCATATGCTTTTACCTGCA R- TCCATCACCCTTCAGAACCTGATCT	268 bp
E. 0.5Kb HindIII 176bp; (probe 7)	F- AAACATGGAACATCCTTGTGGGGAC R- CATTCCTATTAGATCTGTCGCCCTAC	547 bp
F. 1.2/3.8Kb HindIII 186bp; (probe 8)	F- TTGAATACATTGGTTAAATCCCAACATG R- CCTGAATAAAGTCTTCCTTACCACAC	506 bp
G. Exon 12 ???bp; (probe 2)	F- GATAGTGGGCTTTACTTACATCCTTC R- GAAAGCACGCAACATAAGATACACCT	331bp
H. 3.1kb Hind III ???bp; (probe 8)	F- GAAATTGGCTCTTTAGCTTGTGTTTC R- GGAGAGTAAAGTGATTGGTGGAAAATC	388 bp
I. Exon 4 ??bp; (probe 1a)	F- TTGTCGGTCTCCTGCTGGTCAGTG R- CAAAGCCCTCACTCAAACATGAAGC	196 bp

1 Each exon is designated a letter. When known, the exon number is listed; when not known the size of the genomic Hind III fragment that the exon is located on is listed. Also indicated is the human DMD cDNA probe that hybridizes with each exon (4), as well as the size of the exon in base pairs (bp).

2 Shown is the sequence in 5'-3' orientation for the PCR primers used to amplify each region. F: forward primer, hybridizes 5' of the exon; R: reverse primer, hybridizes 3' of the exon.

3 The size of the amplified fragment obtained with each primer set.

Exhibit 2

Technical Comparison of the DMD Mulitplex Protocol to Claims 1, 20, 21 and 22 of Caskey et al. U.S.S.N. 315,673

CLAIM 1	PROTOCOL
A method for simultaneously detecting known deletions from at least three DNA sequences,	The DMD Protocol is a method for detecting known deletions from nine DNA sequences (see the Figure and Table 1 of Protocol)
Comprising the steps of:	
treating said DNA to form single-stranded complementary strands;	The DMD Protocol (step 6) results in formation of single stranded complementary strands of target DNA
adding at least three pairs of oligonucleotide primers, each pair specific for a different sequence, one primer of each pair substantially complementary to a part of the sequence in the sense-strand and the other primer of each pair substantially complementary to a different part of the same sequence in the complementary anti-sense strand;	The DMD protocol uses nine pairs of oligonucleotide primers, each for a different sequence. Each primer pair meets the sense/anti-sense complementarity requirements of Claim 1.
annealing the at least three paris of primers to their complementary sequences, all primers being subjected to the same reaction conditions;	The DMD protocol anneals the primer pairs to their complementary sequences, and all of the primer pairs are subjected to the same reaction conditions in step 6.
simultaneously extending said at least three pairs of annealed primers from each primer's 3' terminus to synthesize an extension product complementary to the strands annealed to each primer, said extension products, after separation from their complement, being capable of serving as templates for the synthesis of an extension product from the other primer of each pair;	The DMD Protocol (step 6) accomplishes primer extension in the manner required by Claim 1, and the extension product of each primer in a given pair serves as a template for synthesis of extension products from the other primer of the pair.
separating said extension products from said templates to produce single-stranded molecules;	DMD Protocol (step 6) includes separation of the extension products from the templates to produc single stranded molecules.
amplifying said single stranded molecules by repeating, at least once said annealing, extending and separating steps; and	DMD Protocol (step 6) performs amplification by repeating the annealing, extending, and separating steps 23 times
identifying said amplified extension products from each different sequence.	The DMD protocol accomplishes the identification step of Claim 1 as a result of steps 7 and 8 of the Protocol.

CLAIM 20	PROTOCOL
A method for simultaneously detecting at least three DNA sequences,	The DMD Protocol is a method for detecting known deletions from nine DNA sequences (see the Figure and Table 1 of Protocol)
Comprising the steps of:	
adding to a common reaction vessel containing a sample mixture of at least three distinct, target sequences in single-stranded form, at least three pairs of oligonucleotide primers, each pair specific for a different sequence, one primer of each pair substantially complementary to a part of the sequence in the sense-strand and the other primer of each pair substantially complementary to a different part of the same sequence in the complementary anti-sense strand;	The DMD accomplishes this step of Claim 20 in steps 1-3 of the Protocol. The Protocol uses nine primer pairs. The DMD protocol uses nine pairs of oligonucleotide primers, each for a different sequence. Each primer pair meets the sense/anti-sense complementarity requirements of Claim 20.
annealing the at least three pairs of primers to their complementary sequences, all primer being subject to the same reaction conditions;	The DMD protocol anneals the primer pairs to their complementary sequences, and all of the primer pairs are subjected to the same reaction conditions in step 6.
simultaneously extending said at least three pairs of annealed primers from each primer's 3' terminus to synthesize an extension product complementary to the strands annealed to each primer, said extension products, after separation from their complement, being capable of serving as templates for the synthesis of an extension product from the other primer of each pair;	The DMD Protocol (step 6) accomplishes primer extension in the manner required by Claim 20, and the extension product of each primer in a given pair serves as a template for synthesis of extension products from the other primer of the pair.
separating said extension products from said templates to produce single-stranded molecules;	The DMD Protocol (step 6) includes separation of the extension products from the templates to produc single stranded molecules.
amplifying said single stranded molecules by repeating, at least once said annealing, extending and separating steps; and	The DMD Protocol (step 6) performs amplification by repeating the annealing, extending, and separating steps 23 times
identifying whether amplified extension products have been synthesized from each different sequence, as a result of the presence or absence of each target sequence.	The DMD protocol accomplishes the identification step of Claim 20 as a result of steps 7 and 8 of the Protocol.

CLAIM 21	PROTOCOL
A method for simultaneously detecting known deletions from at least three DNA sequences,	The DMD Protocol is a method for detecting known deletions from nine DNA sequences (see the Figure and Table 1 of Protocol)
Comprising the steps of:	
treating said DNA to form single-stranded complementary strands;	The DMD Protocol (step 6) results in formation of single stranded complementary strands of target DNA
adding at least three pairs of oligonucleotide primers, each pair specific for a different sequence, one primer of each pair substantially complementary to a part of the sequence in the sense-strand and the other primer of each pair substantially complementary to a different part of the same sequence in the complementary anti-sense strand and each primer having a Tm such that the lowest Tm and highest Tm of all added primers varies by no more than 8.3° C;	The DMD accomplishes this step of Claim 21 in steps 1-3 of the Protocol. The Protocol uses nine primer pairs. The DMD protocol uses nine pairs of oligonucleotide primers, each for a different sequence. Each primer pair meets the sense/anti-sense complementarity requirements of Claim 21. The nine primer pairs used in the DMD protocol are such that the lowest Tm and highest Tm of all eighteen primers varies by no more than 8.3°C.
annealing the at least three pairs of primers to their complementary sequences, all primer being subject to the same reaction conditions:	The DMD protocol anneals the primer pairs to their complementary sequences, and all of the primer pairs are subjected to the same reaction conditions
simultaneously extending said at least three pairs of annealed primers from each primer's 3' terminus to synthesize an extension product complementary to the strands annealed to each primer, said extension products, after separation from their complement, being capable of serving as templates for the synthesis of an extension product from the other primer of each pair;	The DMD Protocol (step 6) accomplishes primer extension in the manner required by Claim 21, and the extension product of each primer in a given pair serves as a template for synthesis of extension products from the other primer of the pair.
separating said extension products from said templates to produce single stranded molecules;	The DMD Protocol (step 6) includes separation of the extension products from the templates to produce single stranded molecules.
amplifying said single stranded molecules by repeating, at least once said annealing, extending and separating steps; and	The DMD Protocol (step 6) performs amplification by repeating the annealing, extending, and separating steps 23 times
identifying and measuring said amplified extension products from each different sequence to detect said known deletions.	The DMD protocol accomplishes the identification step of Claim 21 as a result of steps 7 and 8 of the Protocol.

CLAIM 22	PROTOCOL
A method for simultaneously detecting the presence or absence of at least three DNA target sequences,	The DMD Protocol is a method for detecting the presence or absence of nine target sequences (see the Figure and Table 1 of the Protocol)
Comprising the steps of:	
adding to a common reaction vessel containing a sample mixture of at least three distinct, target sequences in single-stranded form, at least three pairs of oligonucleotide primers, each pair specific for a different sequence, one primer of each pair substantially complementary to a part of the sequence in the sense-strand and the other primer of each pair substantially complementary to a different part of the same sequence in the complementary anti-sense strand and each primer having a Tm such that the lowest Tm and highest Tm of all added primers varies by no more than 8.3° C;	The DMD accomplishes this step of Claim 22 in steps 1-3 of the Protocol. The Protocol uses nine oligonucleotide primer pairs, each pair for a different sequence. Each primer pair meets the sense/anti-sense complementarity requirements of Claim 22. The nine primer pairs used in the DMD protocol are such that the lowest Tm and highest Tm of all eighteen primers varies by no more than 8.3°C.
annealing the at least three pairs of primers to their complementary sequences, all primer being subject to the same reaction conditions;	The DMD protocol anneals the primer pairs to their complementary sequences, and all of the primer pairs are subjected to the same reaction conditions in step 6.
simultaneously extending said at least three pairs of annealed primers from each primer's 3' terminus to synthesize an extension product complementary to the strands annealed to each primer, said extension products, after separation from their complement, being capable of serving as templates for the synthesis of an extension product from the other primer of each pair;	The DMD Protocol (step 6) accomplishes primer extension in the manner required by Claim 22, and the extension product of each primer in a given pair serves as a template for synthesis of extension products from the other primer of the pair.
separating said extension products from said templates to produce single stranded molecules;	The DMD Protocol (step 6) includes separation of the extension products from the templates to produce single stranded molecules.
amplifying said single stranded molecules by repeating, at least once said annealing, extending and separating steps; and	The DMD Protocol (step 6) performs amplification by repeating the annealing, extending, and separating steps 23 times
identifying whether amplified extension products have been synthesized from each different sequence, as a result of the presence or absence of each of said DNA target sequences.	The DMD protocol accomplishes the identification step of Claim 22 as a result of steps 7 and 8 of the Protocol.

CURRICULUM VITAE

March 1995

A. Personal

Name:	Julian Gordon
Born:	February 10, 1936, Chingford, Essex, U.K.
Nationality:	Naturalized US citizen, as of November 25, 1992
Family Status:	Married, 2 children ages 22 and 25
Wife:	Karin Bente, Naturalized US, as of November 25, 1992
Home Address	307 East Sheridan Road Lake Bluff, IL60044
Phone:	(708) 937 7609 (708) 234 2089
FAX	(708) 937 0341
Internet:	gordonj%amgate@matrx.abbott.com

B. University Training

B.Sc. (Special Physics, Upper Second Class Honours).
University of London, Kings College, 1957.

Ph.D. (Biophysics). University of London, Kings College,
Department of Physics and M.R.C.. Biophysics Research Unit.
Thesis Advisor, Dr. G.L. Brown, awarded 1963.

C. Positions and Awards

State Scholarship, 1954-1957.
M.R.C. Postgraduate Scholarship, 1957-61.
Demonstratorship in Physics, Kings College, 1957-61.
NATO Postdoctoral Fellowship, held at University Microbiology Institute, Copenhagen, 1961-63.
Research Associate, University Biochemistry Institute, Uppsala, Sweden, 1963-65.
Research Associate, Rockefeller University, New York, 1965-68
Assistance Professor, Rockefeller University, 1968-71.
Group Leader, Friedrich Miescher Institute, Basel, Switzerland, 1971-84.
Member of European Molecular Biology Organization, 1975 to present.
Senior Immunologist, Abbott Laboratories, North Chicago, 1984 to 1986
Associate Research Fellow, Volweiler Society, 1986 to 1988
Research Fellow, Volweiler Society, 1988 to 1993
Senior Research Fellow, Volweiler Society, 1993—
Sarstedt Prize, awarded in Vienna, Austria, February, 1988
Abbott Researcher of the Year Award, 1992

D. Teaching and Related Experience

Demonstratorship to undergraduates during graduate studies.
Organization of weekly departmental seminars at Rockefeller University, 1967-71.
Organization of internal research seminars at FMI, 1972-75.
Organization and participation in lecture series on "Ribosomes and Protein Synthesis", University of Basel, 1974.
Lectures on "Protein Synthesis", Mode of Action of Antibiotics which Affect Protein Synthesis", University of Basel, 1978.
Research Advisor to postgraduate students:

L. Isaksson	1963-1965
I. Krisko	1967-1969
M. Geiser	1974-1978
H-P. Ramjoue	1976-1978

E. Languages

Mother Tongue: English
Foreign Languages: German, French, Danish

F. Concise Scientific Biography

Ph.D. from London University for work on bacteriophage and bacterial DNA physical chemistry under Dr. G.L. Brown, Biophysics Research Unit.

1961-1965

Postdoctoral and Fellowships at University of Copenhagen, Denmark, under Prof. Maaløe and University of Uppsala, Sweden under H.G. Boman; work on ribosome characterization and **first demonstration of ribosomal RNA methylation *in vitro*.**

1965-1971

Faculty position at Rockefeller University under Fritz Lipmann where work was done on the mechanisms of chain elongation in protein synthesis and **first demonstration of intermediate complexes between tRNA and elongation factors.** Beginning of work with immunological characterization of chain elongation factors, and use of antibodies in showing in vivo regulation in bacteria. **First demonstration of interchangeability of these elongation factors between prokaryotes and eukaryotes.**

1971-1984

Group Leader at FMI in Basel, where important work was done using immunological probes for functional characterization of individual ribosomal proteins. Use of immunological probes to characterize evolutionary relationships in ribosomal proteins. Characterization of conformers of ribosomal RNA. Use of cross linking reagents in attempt to develop novel interferon inducers. **First demonstration of in vivo correlation between ribosomal protein phosphorylation and increased translational activity. First demonstration of Western Blotting on nitrocellulose, and use in the establishment of first hybridomas specific for eucaryotic ribosomal proteins. First demonstration of dot immunobinding assays for screening monoclonals and for screening pathological sera.**

G. Marketed Products

Western Blot, now widely used as AIDS confirmatory test. Dot Immunobinding led to the Abbott Matrix product line: Allergy tests, HIV confirmatory tests. Immunochromatographic membrane technology led to Abbott Test Pack Plus product line: hCG, Strep A, Chlamydia

H. Current Interests and responsibilities.

Development of DNA probe based delivery technology for diagnosic tests, and development of concepts for future technology for diagnostic tests. Heading a group working on developing of a prototype system for determining the presence of human mutations, the system being capable of analysing multiple genetic loci simultaneously and utilizing a completely contained disposable.

I. Hobbies/Interests

Running; completed 7 marathons in last 3 years; running competitively distances from 5K to marathon; cross-country skiing; desk top publishing; data base searching for information in areas of science, medicine, patents, business, news. Editor of Lake Forest/Lake Bluff Running Club Newsletter, 1989-92. Student pilot.

J. Bibliography

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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TRANSMITTAL LETTER

Dear Sir:

Attached hereto for filing in the above-identified application are the following:

- Petition for Acceptance of Photographs Under § 1.84(b)
- Letter to the Official Draftsman

Applicant believes that $130 is due under 37 C.F.R. § 1.17(h) to accept photographs under C.F.R. § 1.84(b). Please charge the required fee for accepting photographs and any other additional fees which are due to the Deposit Account of Fulbright & Jaworski L.L.P., Account No. 06-2375, under order No. 883159/D-5050-3C. An additional copy of this letter is attached for accounting purposes.

Respectfully submitted,

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